Distribution of HIV in different cell types *in vivo* : Implications for pathogenisis.

By: Wendy Livingstone

Faculty of Medicine
Department of Medical Microbiology
University of Edinburgh
Teviot Place
Edinburgh, EH8 9AG

Submitted: October 1997

TO MY MUM, DAD, RICHARD
AND MY SISTER DEBBIE.

DECLARATION

The results in this thesis and its composition are soley the work of the author unless otherwise stated.

ACKNOWLEDGEMENTS

I wish to thank the following people for their help and support:-

Dr Peter Simmonds for his advice, discussion and supervision over the past three years.

Dr Marilyn Moore, Dr Jeanne Bell, Dr Donald Smith and Dr Lisa Jarvis for their support and discussion,

Carol, Alison, and Sarah and all the members of the HCV research group for their endless support.

thanks to Linda and Katrina for the thesis self help group.

Donald Innis and Helen Mason for their help in carrying out FACS analysis.

the Medical Research Council for their financial support.

my parents, Debbie, Richard and all my family for their support and encouragement over the last three years.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Ab	Antibody
ADCC	Antibody dependent cell mediated cell cytotoxicity
AIDS	Aquired immunodeficiency syndrome
AIDS DI	AIDS defining illness
Ag	Antigen
AMV	Avian myeloblastosis virus
APCs	Antigen presenting cells
ARC	AIDS related complex
ATP	Adenosine tri-phosphate
C1-C5	Conserved domains 1 to 5 of the envelope glycoprotein
CA	Capsid antigen
CD4	Cluster determinant 4
CDC	Centres for Disease Control
cDNA	Copy DNA
CDR	Complementarity determining region
CNS	Central nervous system
cPPT	Central polypurine track
CTLs	Cytotoxic T lymphocytes
CTS	Central termination signal
DC	Dendritic cell
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Dideoxyribonucleic acid
Dnase	Dideoxyribonuclease
dNTPs	Dinucleotide tri-phosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Env	The virus envelope

env gp	Envelope glycoprotein
ER	Endoplasmic reticulum
ER-ve	E-rosette negative
ER+ve	E-rosette positive
FACS	Fluorescence activated cell sorting
Fc	Fragment crystalline
FCS	Foetal calf serum
gp41	Transmembrane envelope glycoprotein
gp120	External envelope glycoprotein
GM-CSF	Granulocyte-macrophage colony stimulating factor
Het	Heterosexual contact
HIV	Human immunodeficiency virus
HLA	Human leucocyte group A
Homo	Homosexual male
HTLV-III	Human T lymphotropic virus type III
IFN	Interferon
Ig	Immunoglobulin
IL-2	Interleukin 2
IN	Integrase
IVD	Intravenous drug use
Kb	Kilo-base
KDa	Kilo-Daltons
LAV	Lymphadenopathy associated virus
LTR	Long terminal repeat
MA	Matrix protein
mAb	Monoclonal Antibodies
MACS	Magnetic activated cell sorting
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory factor
Min	Minutes

MRC	Medical Research Council
mRNA	Messenger RNA
NC	Nucleocapsid protein
Nef	Negative Factor
NK	Natural killer cell
NSI	Non-syncytium inducing
OC	Oesophageal candidosis
ORF	Open reading frame
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCP	*pneumonocystis carinii* pneumonia
PCR	Polymerase Chain reaction
PGL	Persistent generalised lympadenopathy
PHA	Phytohaemaglutinin
PND	Principal neutralising domain
PPT	Polypurine track
PR	Protease
R	Repeat region
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RRE	Rev responsive element
RT	Reverse transcriptase
RTC	Regional Transfusion Centre
Sec	Seconds
SDF	Stromal cell derived factor
SEB	Staphylococcal enterotoxin B
SI	Syncytium Inducing
SIV	Simian immunodeficiency virus
snRNPs	Small ribonuclear proteins

SRBCs	Sheep red blood cells
STLV-III	Simian T lymphatropic virus type III
Taq	*Thermus aquaticus*
TAR	Tat response element
Tat	Transcription transactivator
TCR	T cell receptor
Tfi	*Thermus flavus*
TNF	Tumour necrosis factor
tRNA	Transfer RNA
TSST-1	Toxic shock syndrome toxin-1
V1-V5	Hypervariable domains of the envelope glycoprotein
Vif	Viral infectivity factor
VPR	Viral protein R
VPU	Viral protein U
VPX	Viral protein X
ZDV	Zidovudine

ABSTRACT

Although CD4 lymphocytes have been identified as the principal target of the human immunodeficiency virus (HIV), the extent to which infection of other cell types of the immune system contributes to the observed impairment in the immune response is unknown. This study investigated the cell types infected with HIV in peripheral blood, and the relation of virus load in different cell subsets with disease progression. Two magnetic cell separation techniques were used to purify cell subsets from 34 HIV seropositive individuals at different stages of disease progression. HIV proviral sequences within these separate cell populations were quantified by limiting dilution nested polymerase chain reaction (PCR). HIV-1 DNA was detected in CD4 and CD8 lymphocytes, monocytes, natural killer cells and dendritic cells. The contribution to total proviral load by different subsets of peripheral blood mononuclear cells (PBMCs) was estimated by quantitative PCR combined with measurements of their relative frequency in peripheral blood. This analysis revealed that CD8 T lymphocytes are a major reservoir of HIV within the peripheral blood of individuals with AIDS.

Further independent evidence for *in vivo* infection of CD8 lymphocytes was obtained by sequence comparisons of the V3 region of the envelope gene. Numerous sequences were obtained from each purified cell subset and initial sequence comparisons indicate that different virus populations may be present in CD4 and CD8 lymphocytes. The functional significance of this will remain unclear

until such variants can be characterised virologically. A PCR based method was developed to detect spliced HIV mRNA transcripts whose presence within cells indicates active viral replication, and differentiates these from cells containing an inactive provirus. High levels of mRNA expression were detected in CD4 lymphocytes, CD8 lymphocytes and in one case in monocytes. This evidence indicates that mature CD8 lymphocytes can be actively infected with HIV-1 *in vivo* even although they do not express CD4.

This study provides evidence for widespread infection of CD4-negative cells within the peripheral blood of HIV seropositive individuals, indicating that HIV-1 has a broader cell tropism for different cell types *in vivo* than described previously. Active infection of not only CD4 T lymphocytes, but also CD8 T lymphocytes, natural killer cells and dendritic cells may contribute to the observed decline of these cell subsets upon disease progression and provide novel mechanisms underlying the immunodeficiency observed in HIV-infected individuals.

CHAPTER 1 : INTRODUCTION

INTRODUCTION

1.1 Background

In 1981 there were reports of an unusually high number of young homosexual males presenting with immunological abnormalities, often accompanied by opportunistic infections, neurological disorders, and unusual forms of cancer. Epidemiological studies suggested that an infectious agent was transmitted through intravenous drug use, blood transfusion, blood products, vertically from mother to child and during sexual intercourse. A retrovirus was isolated from lymph node cells of a patient with lymphadenopathy, lymphadenopathy associated virus (LAV) (Barre-Sinoussi *et al.,* 1983). In the following year a virus described as HTLV-III (Human T-lymphotropic virus type III) was isolated from patients with acquired immunodeficiency syndrome (AIDS) (Gallo *et al.,* 1984). In 1985 a lentivirus, STLV-III (Simian T-lymphotropic Virus type III) was isolated from captive Asian macaques with an AIDS like disease (Daniel *et al.,* 1985). It was demonstrated by electron microscopy that LAV and HTLV-III were morphologically similiar to members of the lentivirus genus of the family *Retroviridae.* Comparisons of genome sequences suggested these viruses were related to lentiviruses of other animals. LAV and HTLV-III were renamed human immunodeficiency viruses (HIV). For the AIDS associated virus in macaques the designation simian immunodeficiency virus (SIV) was adopted. Two distinct types of HIV have been recognised, HIV-1 and HIV-2.

The *Retroviridae* comprise a large family of viruses, and comparison of nucleotide sequence relationships and genome structure suggest that they belong in seven distinct genera (Table 1.1) (reviewed in Coffin, 1996). Retroviruses have several unique features including a diploid RNA genome that encodes the enzyme reverse transcriptase (RNA-dependent DNA polymerase).

Table 1.1 : Retrovirus Genera

Genus	Examples
Avian-leukosis-sarcoma	Rous sarcoma virus (RSV) Avian myeloblastosis virus (AMV) Avian erythroblastosis virus (AEV) Avian myelocytomatosis virus (MC) Rous-associated virus (RAV)
Mammalian C-type	Moleney murine leukemia virus (Mo-MlV) Harvey murine sarcoma virus (Ha-MSV) Abelson murine leukemia virus (A-MuLV) Feline leukemia virus (FeLV)
B-Type viruses	Mouse mammary tumor virus (MMTV)
D-Type viruses	Mason Pfizer monkey virus (MPMV)
HTLV-BLV group	Human T-cell leukemia (or lymphotropic virus) (HTLV)-1 and -2
Lentivirus	Human immunodeficiency virus-1 and -2 Simian immunodeficiency virus (SIV) Feline immunodeficiency virus (BIV)
Spumavirus	Simian foamy virus (SFV) Human foamy virus (HFV) Feline Syncytium-forming virus (FeSV)

Their life cycle involves conversion of the genomic RNA into a double stranded DNA intermediate by the virion-associated reverse transcriptase followed by mandatory integration of the viral DNA into the host chromosome, a step carried out by the viral integrase. HIV-1, HIV-2, and SIV belong to the genus lentiviruses. This genus includes complex exogenous viruses responsible for a variety of neurological and immunological diseases, but not directly implicated in malignancies.

HIV infection involves firstly an acute phase including a transiently high viral load and in some cases a flu-like seroconversion illness. Antibody (Ab) can be detected from 1 to 10 weeks following exposure. Following seroconversion there is generally a sharp reduction in the level of virus that can be detected in the plasma. Seroconversion is followed by virus dissemination in lymphoid tissues and by continuous virus replication in infected cells. During infection there is a correlation between disease progression and the presence of increasing amounts of infectious virus, in the number of infected cells that express viral gene products and a decline in CD4 lymphocytes. This asymptomatic phase can last for several months to more than 15 years. Eventually almost all individuals become immunodeficient presumably because of the low levels of CD4 T cells. This stage of the disease, AIDS, is associated with immune abnormalities, opportunistic infections, neurological disorders, weight loss and unusual forms of cancer.

1.2 Classification of human immunodeficiency viruses (reviewed in Luciw, 1996)

Independent isolates of HIV-1 display the greatest sequence variation in the *env* gene, which encodes the viral envelope glycoprotein. Based on *env* gene sequences, nine subtypes (clades) of HIV-1 (A through I) and five subtypes of HIV-2 have been classified. Within each subtype there is a high degree of variability. Although mutation is a major factor responsible for viral variation, recombination has been postulated to occur in individuals infected with viruses from different clades of HIV-1 and HIV-2. Some areas of the world harbor predominantely a single subtype, whereas two or more subtypes may be prevalent in certain other populations. Molecular epidemiological surveys indicate that the current global distribution of subtypes is due to viral migration rather than to viral mutation.

HIV-1 viruses are found in several regions of Africa, Asia, Europe, and both North and South America. HIV-2 is a distinct but genetically related virus prevalent in West African countries. Although both cause AIDS, individuals infected with HIV-2 exhibit a longer period of clinical latency and lower morbidity. At present, the origins of HIV-1 and HIV-2 infection in man remain unclear, although it is possible that both of these viruses arose from zoonotic transmissions from nonhuman primates.

1.3 Clinical Aspects of HIV-1 Infection

Infection with HIV-1 results in a range of clinical conditions from asymptomatic infection to severe immunodeficiency resulting in opportunistic infections and neoplasms. A number of classification systems for HIV-1 related illnesses have been proposed. The most recent Centres for Disease Control (CDC) system classifies the manifestations of HIV-1 into three clinical categories, A, B, and C (table 1.2) (reviewed in Hirsch *et al.*, 1996).

Table 1.2 : 1993 revised classification system for HIV infection and expanded AIDS surveillance case definition for adolescents and adults

	Clinical Categories		
CD4 T-cell categories[b]	(A) Asymptomatic acute HIV or PGL[a]	(B) Symptomatic, not (A) or (C) conditions	(C) AIDS-indicator conditions
(1) >500/μl	A1	B1	C1
(2) 200-499/μl	A2	B2	C2
(3) <200/μl (Aids Indicator)	A3	B3	C3

a PGL : Persistent generalised lymphadenopathy
b CD4 T-cell categories correspond to CD4 T lymphocytes per μl blood.

(i) Acute Primary Infection Syndromes (Clinical category A)

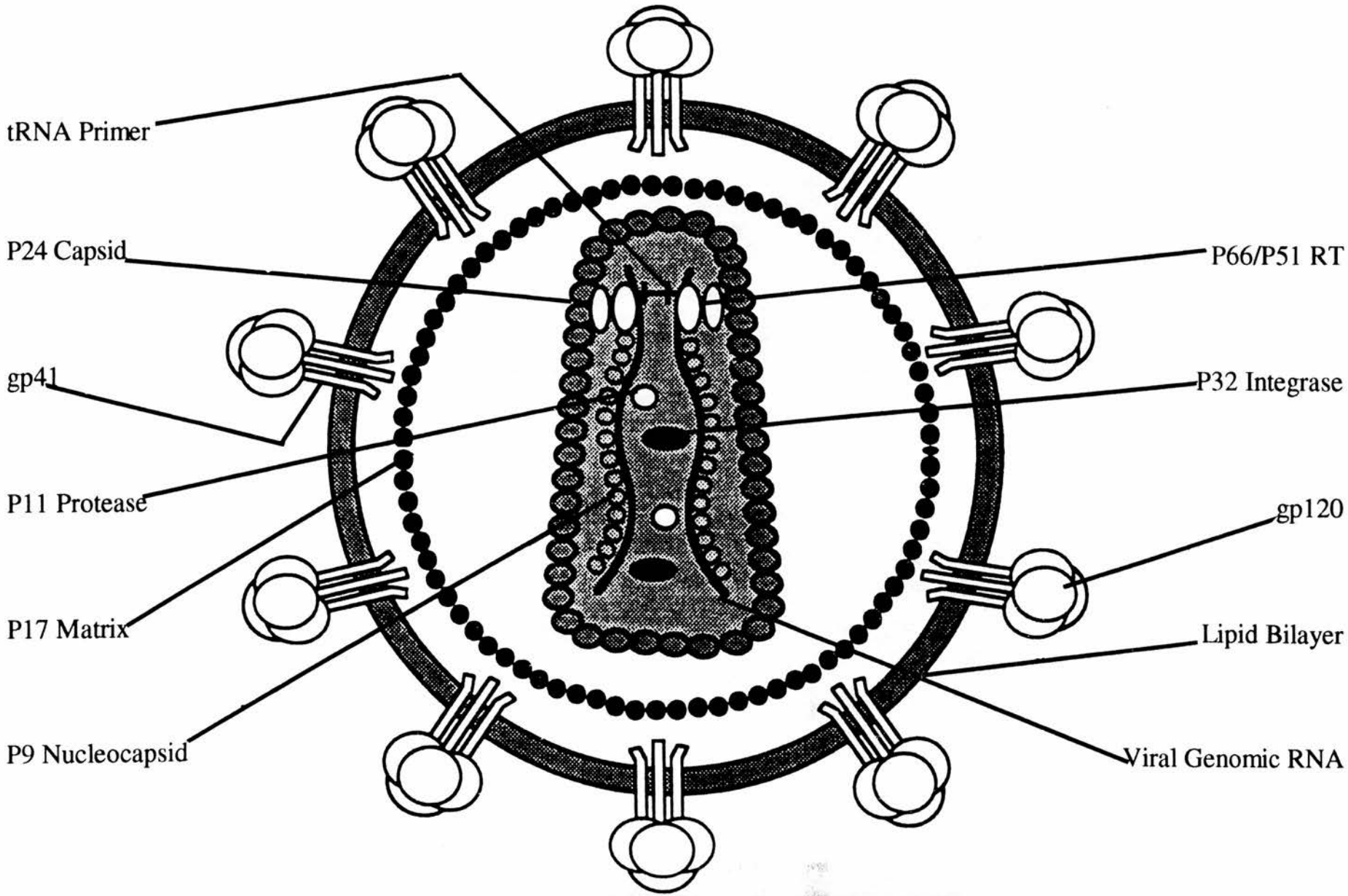
Some HIV-1 seroconversions are asymptomatic while others are associated with a self-limited mononucleosis or influenza-like syndrome characterised by fever,

rigors, arthralgias, myalgias, malaise, lethargy, anorexia, nausea, diarrhoea, sore throat and a urticarial or vesicular rash. Neurological symptoms including headaches, a stiff-neck, irritability and depression may also be evident. This illness may last 2 to 3 weeks but usually results in recovery. Incubation periods ranging from a few days to 3 months have been described. During primary infection plasma HIV-1 titres increase generally for a few weeks until a specific antibody response is mounted.

(ii) Asymptomatic Infection and Persistent Generalised Lymphadenopathy (Clinical Category A).

In adults the mean period of asymptomatic infection before the development of AIDS has been estimated at 10 years in the absence of therapy. Within the HIV-1 seropositive population CD4 lymphocyte counts give an indication of the degree of immunosuppression and predict the risk of developing AIDS. Evidence suggests that the risk of developing AIDS in individuals with CD4 counts less than 200/μl is much greater than in those individuals with higher CD4 counts. More recently plasma viral load measurements have become more accurate due to the development of PCR techniques and these measurements are also useful in predicting disease progression. CD4 counts and viral load measurements have also proved effective in determining an individuals response to anti-retroviral therapy.

Figure 1.1 : The HIV-1 virion structure (Modified from Fields Virology, 1996)


tRNA Primer
P24 Capsid
gp41
P11 Protease
P17 Matrix
P9 Nucleocapsid
P66/P51 RT
P32 Integrase
gp120
Lipid Bilayer
Viral Genomic RNA

(iii) Symptomatic HIV Infection (Clinical Categories B and C)

After a variable period of asymptomatic HIV-1 infection a variety of symptoms indicate clinical deterioration including chronic fevers, night sweats, diarrhoea, weight loss, herpes zoster, oral thrush (OC), hairy leukoplakia, cytomegalovirus disease, encephalopathy, or *pneumonocystis carinii* pneumonia (PCP). The term AIDS-related complex (ARC) is used when two or more symptoms or two or more laboratory findings are indicative of immune dysfunction. Once the diagnosis of AIDS has been made, survival is often less than two years, although considerable variability exists depending on various factors such as age and therapy.

1.4 Virion Structure

The HIV-1 virion is spherical in shape with a diameter of approximately 110 nm (figure 1.1) (reviewed in Levy, 1993; Luciw, 1996). A lipid bilayer envelope surrounds a cone shaped nucleocapsid which is connected at the narrow end to the lipid bilayer. The region between the viral envelope and nucleocapsid has been termed the paranucleoid region, core shell, or lateral body. Each mature virion contains two molecules of single-stranded RNA genome encapsulated by proteins that have been proteolytically processed from the Gag precursor polypeptide. These proteins are: the matrix protein (MA), located between the nucleocapsid and the virion envelope; the major capsid protein (CA) which forms the capsid shell; and the nucleocapsid protein (NC) which binds tightly to the RNA genome. The

5' ends of the genome are base paired. A transfer RNA (tRNA) molecule is positioned near the 5' end of each genomic RNA strand and serves as the primer for initiation of negative strand viral DNA synthesis by reverse transcriptase (RT). Several enzymes derived from the *pol* gene precursor polypeptide are also packaged into virions; protease (PR), reverse transcriptase (RT), and integrase (IN). Viral protein R (Vpr) (and Vpx for SIV and HIV-2) are small proteins associated with the nucleocapsid.

HIV particles have approximately 72 heterodimers of the envelope glycoprotein (Env gp), each of these is composed of a surface subunit (gp120) that interacts with the transmembrane subunit (gp41) through noncovalent bonds.

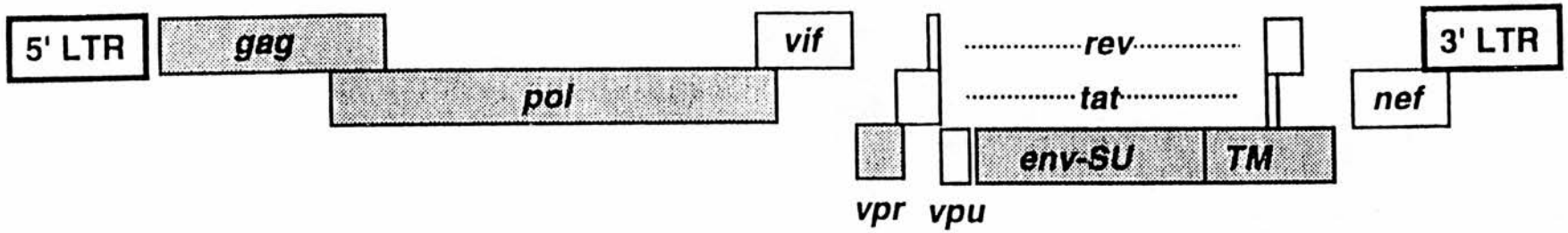
1.5 Viral Genome Structure and Organisation (reviewed in Levy, 1993; Coffin, 1996; Luciw, 1996)

Infectious virions of HIV and SIV contain two copies of single stranded RNA of positive polarity in respect to translation. In the early steps of replication the virus RNA is converted into double stranded DNA which is then integrated into the host genome. The reverse transcription and synthesis of double stranded DNA results in the formation of two identical long terminal repeats (LTRs) flanking the viral genes. Therefore HIV and SIV have two genetic forms, single stranded RNA in virions and double stranded DNA (provirus) within the cell.

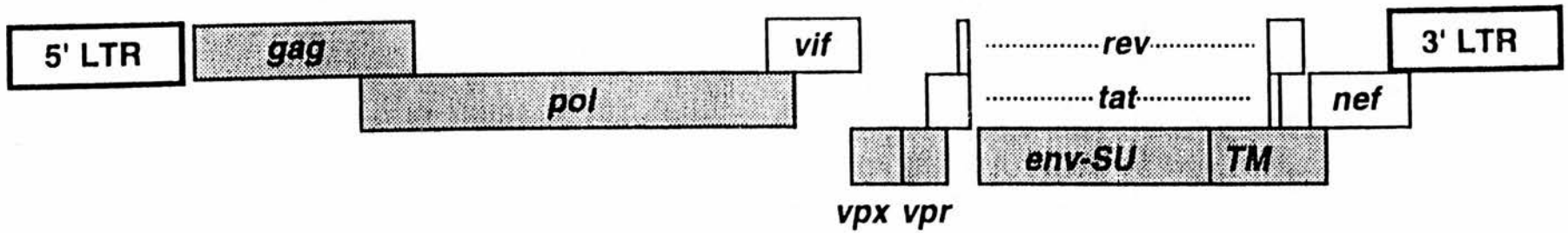
Most retroviruses contain three long, continous open reading frames (ORFs) coding for the Gag, Pol and Env proteins. However, the genomic organisation of

Figure 1.2 : Genomic Organisation of HIV-1 and HIV-2/SIV_{MAC} (From Fields Virology 1996). *env*-SU = gp120 the external envelope glycoprotein. TM = gp41, the transmembrane envelope glycoprotein.

HIV-1



HIV-2 / SIV_{MAC}



lentiviruses, including HIV-1, is more complex (figure 1.2) and through the use of ribosomal frame shifting, alternative splice sites, and bicistronic RNAs, the HIV genome encodes three structural proteins Gag, Pol, and Env, and six auxiliary proteins, Tat, Rev, Vif, Vpr, Vpu, and Nef. Expression of the HIV-1 genome is mediated by three classes of mRNA: unspliced (9.0 kb), single-spliced (4.5 kb) and multiply spliced, (1.8-2.2 kb). The structural protein precursors Gag and Pol are produced from unspliced RNAs, while Env, Vif, Vpr and Vpu proteins are encoded by singly spliced mRNA. Other regulatory genes are expressed from multiply spliced mRNAs.

Mutations affecting either Tat or Rev severely impair viral replication but mutations affecting the other auxiliary proteins do not generally perturb the viral replication kinetics, at least *in vitro* and are usually referred to as accessory gene products.

1.6 Viral Proteins (Reviewed in Levy, 1993; Coffin, 1996; Luciw, 1996)

(i) Core/Capsid Proteins

The *gag* gene is translated into a polyprotein precursor (p55) from an unspliced mRNA and is subsequently cleaved by the viral protease into the mature non-glycosylated capsid proteins of HIV, matrix (MA, p17), capsid (CA, p24), p2, nucleocapsid (NC, p7), p1 and p6.

(a) Matrix (MA, p17)

Mature MA contains about 130 amino acids, with a molecular weight of 17 to 18 kDa and is located in the matrix between the virion capsid and the envelope. The MA protein has a close association with the viral membrane and can be cross-linked to lipid, and has a stretch of basic amino acids resembling the nuclear localisation signals of nucleophilic proteins. MA may be part of the viral nucleoprotein complex produced after reverse transcription and may mediate nuclear import of this pre-integration complex. Deletions in MA also impair envelope glycoprotein incorporation into virions resulting in defective virus particles.

(b) Capsid Protein (CA, p24)

The capsid protein (CA) is released from the central portion of the Gag polyprotein by two cleavages mediated by the viral protease. The mature form of CA contains about 240 amino acids (molecular weight of 24 to 27 kDa), has a high degree of hydrophobicity, and is a major subunit of the capsid shell. The domain governing nucleocapsid assembly is located in the C-terminal portion of CA. Specific interactions of CA with the viral RNA genome and other Gag proteins may also play a role in CA function during capsid assembly.

(c) Nucleocapsid (NC, p7)

Viral protease-mediated cleavage of the C-terminal portion of Gag produces the nucleocapsid (NC) a protein of about 70 amino acids with a molecular weight ranging from 7 to 9 kDa. This basic, hydrophilic protein binds viral genomic RNA in the nucleocapsid and may condense the RNA genome for packaging into capsids. A cysteine-histidine motif present in NC is similiar to the metal-binding finger domains of several proteins that interact with nucleic acids. The cysteine-histidine motif may also influence transfer RNA (tRNA) annealing to the viral genome. NC may be involved in the early stages of viral replication such as virus uncoating and reverse transcription since it is an intrinsic part of the nucleocapsid. P6, acts later in replication and contains sequences required for efficient release of newly budded virions from the infected cell.

(ii) The Viral Enzymes

The viral enzymes protease (PR, p10), reverse transcriptase (RT) and RNase H (p66/p51) and integrase (IN, p32), are produced by cleavage of the Gag-Pol polyprotein (p160). In HIV the *gag* and *pol* genes overlap by 241 nucleotides with the the *pol* gene is in a -1 reading frame with respect to *gag*. Expression of the *pol* gene is facilitated by a ribosomal frameshift which occurs at a low frequency and is directed by a short sequence located in the overlap between the two genes that allows a 'slip-back' of the ribosome into the -1 position. This results in relatively large amounts of gag transcripts and smaller quantities of the *pol* transcripts.

(a) Protease (PR)

The mature form of the protease (PR) is 99 amino acids in length (molecular weight 10 kDa). For processing, the Gag-Pol polyprotein dimerizes in the infected cell, and the mature PR dimer is released by autocatalytic cleavage. The fully active PR dimer has multiple specificity and targets five cleavage sites in Gag, and seven in the Gag-Pol polyproteins. Site specific mutagenesis demonstrates that PR is essential for replication.

(b) Reverse transcriptase (RT) and RNase H

P66 is cleaved from the Gag-Pol polyprotein and forms a homodimer. Subsequently, one subunit of p66 is cleaved by PR to yield a heterodimer composed of p51 and p66. Both the heterodimer and p66 homodimer display RNase H and RT activity . RT is an RNA-dependent DNA polymerase which produces DNA from both RNA and DNA templates and requires an oligonucleotide primer. RNase H cleaves RNA in RNA DNA hybrids and is necessary for the synthesis of double stranded DNA.

(c) Integrase (IN, p32)

The Gag-Pol precursor polypeptide is proteolytically processed by viral PR to produce a 32 kDa integrase. An integrase domain near the N-terminus contains pairs of histidine and cysteine residues that adopt a structure similiar to the metal-finger motif of DNA binding proteins. Integrase possesses both DNA cleavage and joining (or strand-transfer) activities and mediates covalent linkage of linear double stranded viral DNA into the host cell genome.

(iii) The envelope glycoprotein

A spliced transcript of the HIV-1 genome encodes both Vpu and the precursor polyprotein for the envelope glycoprotein. This bicistronic mRNA is synthesised at a late stage of viral replication and its expression is dependent on the post-transcriptional function of the viral *rev* gene. Extensive glycosylation of the Env precursor polyprotein in the endoplasmic reticulum (ER) and golgi apparatus during synthesis produces gp160. Intracellular cleavage of gp160 yields a 550 amino acid N-terminal subunit (gp120), and a 330 amino acid C-terminal subunit (gp41). The processed Env oligomers are directed out of the golgi compartment and into the plasma membrane.

The envelope glycoprotein is a heterodimer consisting of the gp120 and gp41 subunits held together by noncovalent bonds which are themselves organised into an oligometric complex. High resolution electron microscopy of virions reveals trimeric symmetry of the envelope glycoprotein spikes.

(a) The external envelope glycoprotein (gp120)

gp120 is a highly glycosylated, hydrophilic protein positioned on the external surface of virion membranes as well as on the plasma membranes of infected cells. HIV-gp120 contains 24 potential sites for N-linked glycosylation (Asn-X-Ser/Thr) and 17 of these are modified with carbohydrate side chains. Therefore very few regions of the peptide backbone protrude from the carbohydrate mass. HIV-gp120 has 18 cysteine residues, that are highly conserved in the glycoproteins of diverse HIV-1 and HIV-2 or SIV strains and are presumed to play a critical role in its structure and function. A model for the gp120 subunit, based in part on biochemical analysis, shows nine intra-chain disulphide bonds. This disulphide bonding pattern delineates gp120 into several different regions which include five conserved domains (C1-C5), five hypervariable domains (V1-V5), and a conformation dependent domain that interacts with the CD4 receptor.

(b) The trasmembrane envelope glycoprotein (gp41)

gp41 is a relatively hydrophobic protein that traverses the lipid bilayer membranes of both virions and cells and is classified as a type 1 integral membrane protein. The gp41 sequence contains four potential glycosylation sites and three cysteine residues. A stretch of about 20 amino acids at the N-terminus of gp41 are hydrophobic and are required for the fusion of the virion membrane with the cell plasma membrane during virus entry. A second hydrophobic domain spans the lipid bilayer and enables gp41 to serve as an anchor for gp120. The region between

these two hydrophobic domains is external to the membrane and contains a highly conserved sequence similiar to the leucine zipper motif implicated in protein-protein interactions of a variety of viral and cellular proteins.

(iv) Essential Auxiliary Proteins

The relative levels of different HIV-1 mRNAs are controlled by the viral regulatory factors Tat and Rev, as well as by cellular factors.

(a) Tat (Transcription activator)

Tat is encoded by two exons, one 5' and one within the *env* gene. Tat is a transactivator of LTR-directed gene expression and functions through a cis-acting sequence, designated the Tat response element (TAR), which is located in the repeat (R) portion of the LTR. TAR is present in transcripts and folds into a characteristic stem-loop structure. Several different mechanisms of the Tat/TAR interaction have been proposed. These include bypassing of a transcriptional termination site, stimulation of transcription initiation by simultaneous interaction with the nascent RNA and transcription factors, or combinations of these mechanisms. Tat has also been shown to affect the expression of a number of cellular genes, both positively and negatively.

(b) Rev

Viral gene expression is modulated during HIV replication such that early in infection only multiply spliced mRNA species accumulate within the cytoplasm of infected cells while unspliced and incompletely spliced viral mRNAs remain confined to the nucleus. Only late in infection do the incompletely spliced RNAs accumulate within the cytoplasm and become translated into the viral structural proteins.

Like *tat,* the *rev* gene is composed of two exons, one 5' and one within the *env* gene. The Rev protein binds to a cis-acting RNA target sequence, the Rev responsive element (RRE), so preventing the nuclear sequestration of incompletely spliced viral transcripts. Rev therefore increases the cytoplasmic levels of unspliced and partially spliced HIV-1 mRNAs, resulting in the efficient expression of the viral proteins.

(v) Accessory Proteins

(a) Vif (Viral Infectivity Factor)

The gene encoding Vif is located immediately downstream of the *pol* gene. Vif, a cytoplasmic protein, is synthesised from an open reading frame encoding 193 amino acids (molecular weight 23 kDa). Virions generated in its absence are as much as 1000 times less infectious. In comparison, cell-to-cell infection is only slightly impaired in the absence of Vif, indicating that the defect lies primarily in the progeny virions. Studies have failed to document a notable difference in either

the quantity, processivity, or the apparent molecular weight of envelope and other viral proteins from virions generated in the absence of Vif and the reverse transcriptase from these virions was capable of efficiently initiating DNA synthesis. Vif may therefore act late during virion assembly and be necessary for the proper packing of the viral nucleoprotein core.

(b) Vpr (Viral Protein R)

Vpr is a 14 kDa virion-associated protein translated from a singly spliced mRNA, the product of which is dependent on Rev function and thus accumulates at late times in infection. In mature virions, Vpr is associated with the nucleocapsid and may play a role in an early step of the virus life cycle such as nuclear localisation of the viral pre-integration complex. Vpr-positive strains grow faster and produce moderately higher levels of virus than their Vpr-negative counterparts. This is more pronounced for infection of primary macrophages but not in primary T cells, suggesting that Vpr function may be important in specific target cells. Vpr may influence viral gene expression by altering a host cell regulatory mechanism.

A strong sequence homology exists between the viral protein Vpx of HIV-2 and Vpr of HIV-1. Vpx is also packaged into mature virions and confers a rapid growth advantage.

(c) Vpu (Viral Protein U)

Vpu is a 16kDa protein translated from a singly spliced mRNA dependent on Rev function and produced at late times in infection. The reading frame for Vpu overlaps the 5' end of *env* but in a different reading frame. The absence of Vpu results in virions containing multiple cores and virus budding into vacuolar compartments as opposed to the plasma membrane. Vpu has been demonstrated to degrade CD4 in the endoplasmic reticulum (ER). In infected cells, CD4 is also trapped by the Env gp in the ER and produces gp160-CD4 complexes. Consequently, surface expression of CD4 is downregulated, and cleavage of gp160 to gp120 and gp41 is reduced. By decreasing the stability of intracellular CD4, Vpu reduces the formation of gp160-CD4 complexes and thereby increases the rate of gp160 processing.

(d) Nef (Negative Factor)

Nef, like Tat and Rev, is expressed early in the viral replication, however its function is not essential *in vitro*. The *nef* gene of HIV-1 extends from the 3' end of *env* into the U3 domain of the 3' LTR. Nef is translated from two multiply spliced early transcripts that are independent of the post-transcriptional function of Rev. Initial studies characterised Nef as a down regulator of viral gene expression, but later reports found no Nef mediated transcriptional inhibition of HIV-1 replication, and kinetic studies indicated a positive effect of Nef. Expression of

HIV *nef* genes in CD4 T cells is also associated with a reduction in the level of CD4 receptor on the cell surface.

1.7 The life cycle of HIV-1 (Reviewed in Levy, 1993; Luciw, 1996)

(i) Attachment, Entry and nuclear transport.

Attachment and Entry.

HIV-1 binds to the surface of cells expressing the cellular receptor for HIV-1, CD4 (Dalgleish *et al.*, 1984; Maddon *et al.,* 1986; Klazmann *et al.,* 1984). Sattentau and Moore (1991) demonstrated that when a soluble recombinant form of CD4 complexes with gp120 conformational changes are induced within the envelope glycoprotein oligomers resulting in increased exposure of gp41 epitopes, including the hydrophobic amino terminus of the transmembrane envelope glycoprotein (gp41) allowing entry of the virus into the cytoplasm (Levy 1993). (see section 3.1).

Two studies investigating the crystal structure of the gp41 ectodomain, have revealed a triple-stranded alpha-helical coiled coil region with the amino terminus at its tip within the core. A carboxy-terminal alpha-helix packs against the outside of the coiled coil, placing the amino and carboxy termini near one another at one end of a rod like molecule. The helical regions are separated by an immunodominant region which contains a short loop held together by an intra-molecular disulphide bond (Weissenhorn *et al.,* 1997; Chan *et al* 1997). The crystal structures show that three molecules of each peptide fragment are present in the stable configuration, suggesting that the glycoprotein complex on the HIV-1

envelope is trimeric (Chan *et al.,* 1997). The structure of gp41 is similiar to the low pH induced conformation of influenza hemagglutinin (HA), however unlike influenza viruses HIV-1 fuses at the plasma membrane without a pH change, in a process that involves binding of the virus to CD4 and a chemokine co-receptor (Alkhatib *et al.*, 1996; Choe *et al.*, 1996; Deng *et al.*, 1996; Doranz *et al.*, 1996; Dragic *et al.*, 1996; Feng *et al.*, 1996) (section 3.2).

These crystallized helices represent a post HIV-cell fusion structure, however the pre-fusion structure can be inferred. Binley and Moore (1997) compared the pre-fusion structure to a mousetrap. The helical peptide fragments represent the two halves of the trap, separated by a disulphide loop at the "hinge". A "needle" (the fusion peptide) is attached to one end under tension. Binding to the CD4 receptor brings both helices together and fires the fusion peptide down into the target cell membrane initiating fusion of viral and cell membranes. The binding of HIV-1 to soluble CD4 is enough to cause conformational changes in the viral envelope (Sattentau and Moore, 1991) but these changes create or expose the co-receptor binding sites on gp120 allowing the fusion peptide to reach the cell membrane (Wu *et al.*, 1996; Trkola *et al.*, 1996).

Nuclear Transport: The role of vpr and p17 in nuclear transport in non dividing cells and vpr in cell cycle arrest.

After infection, the preintegration complex (PIC) of HIV-1 is rapidly transported to the nucleus using the host cell active transport processes obviating the requirement of host cell division in establishment of the integrated provirus (Bukrinsky *et al.*, 1992). The ability of HIV-1 to replicate in non-dividing cells is partly due to the karyophilic properties of the uncoated PICs. (Bukrinsky *et al.*, 1993), reflected by the presence of nuclear localisation signals (NLSs) on at least two of its components MA, and Vpr (Bukrinsky *et al.*, 1993; Goldfarb, 1995; von-Schwedler *et al.*, 1995; Mahalingarn *et al.*, 1997).

The HIV-1 MA protein is a viral membrane protein, myristic acid is a primary determinant of Gag protein-membrane interactions (Spearman *et al.*,1997). MA is also involved in the nuclear import of PICs, this requires the transfer of some MA molecules to the maturing core particle during virus assembly (Goldfarb, 1995). Phosphorylation of Gag MA on tyrosine and serine prior to and during virus infection facilitates its dissociation from the membrane (Bukinskaya *et al.*,1996), IN binds the tyrosine phosphorylated C terminus of a subset of MA proteins, thereby recruiting these molecules into the virion core and subsequently into the uncoated viral nucleoprotein complex (Gallay et al., 1995 a and b).

MA contains in its proximal portion a stretch of basic residues that act as a NLS recognised by the importin/karyopherin pathway (Gallay *et al.*, 1996; Bukrinsky *et al.*, 1993). In the absence of a functional *vpr* gene, MA NLS mutant

viruses fail to replicate efficiently in macrophages (Bukrinsky *et al.*, 1993; von-Schwedler *et al.*, 1995), and cannot establish a stable infection in quiescent T lymphocytes (von-Schwedler *et al.*, 1995). The MA NLS is recognised by RchI a member of the karyopherin-alpha family. Evidence suggests that a member of this family participates in docking the HIV-1 nucleoprotein complex at the nuclear pore (Gallay *et al.*, 1996).

The *vpr* gene product of HIV-1 is a virion-associated protein that is essential for efficient viral replication in monocytes and macrophages. Vpr is packaged efficiently into viral particles through interactions with the p6 domain of the Gag polyprotein p55Gag. Vpr regulates the nuclear import of HIV-1 by binding to karyopherin alpha and increasing its affinity for viral NLSs, including the NLS of MA. Virion-packaged Vpr can also arrest infected T cells in the G2 phase. HIV-1 does not replicate in quiescent (G0) T lymphocytes, however it can productively infect T cells arrested in either G1-S or G2 phase of the cell cycle (reviewed in Bukrinsky *et al.*, 1997). Mahalingam *et al* (1997) revealed that nuclear import, virion incorporation and cell cycle arrest are mediated by distinct functional domains of Vpr.

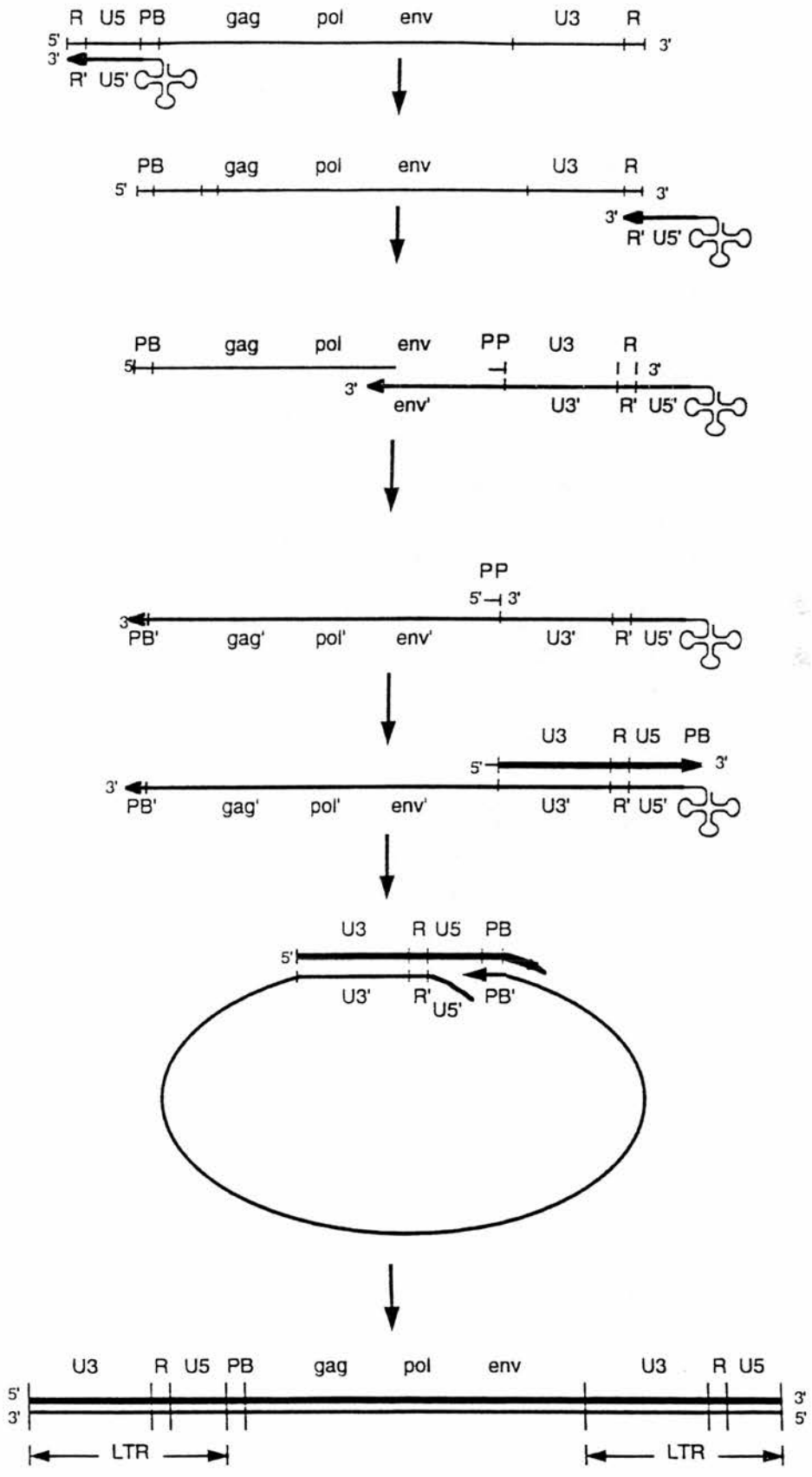
In an early study, Burkrinsky *et al* (1992) reported that HIV-1 integrase was not required for nuclear import. However, in a more recent study, Gallay *et al* (1997), demonstrated that the integrase enzyme can connect the HIV-1 PIC with the cell nuclear import machinery via a NLS-mediated recognition of IN by members of the important/karyopherin-alpha family.

(ii) Reverse Transcription

(a) Synthesis of Minus Strand DNA

Viral particles are partially uncoated immediately after entry into cells to produce a large nucleoprotein complex. HIV-1 has a characteristic molecule of tRNA base paired to the primer binding region near the 5' end of the genome, and reverse transcription is initiated from this primer (figure 1.3). *In vitro* the most prominent early product is strong stop DNA, which is a copy of the short region consisting of R and U5 lying between the primer binding site and the 5' end of the genome. The strong stop DNA, still attached to the tRNA pimer, must transfer, with the reverse transcriptase, to the other end of the genome in order for DNA synthesis to continue. The driving force for "jumping" may be the affinity of the reverse transcriptase enzyme for the template RNA, and the capsid structure rather than sequence complementarity may play a major role in ensuring correct strand transfer. The RNase H activity of reverse transcriptase is also an essential factor in strand transfer as it removes the newly reverse transcribed RNA, leaving the DNA free to pair with the R sequence at the 3' end. Minus strand DNA synthesis then continues on the viral RNA template by the viral RT to the 5' end of the viral RNA genome. Elongation of the growing DNA chain on the RNA template occurs simultaneously with degradation of the template by the RNase H activity of the RT enzyme.

Figure 1.3 : Mechanism of viral DNA synthesis. Thin lines depict RNA; medium lines, minus-strand DNA; thick lines plus-strand DNA. (From Fields Virology 1996)


R U5 PB gag pol env U3 R
5'
3'
R' U5'
PB gag pol env U3 R
5'
3'
3'
R' U5'
PB gag pol env PP U3 R
5'
3'
env'
U3' R' U5'
PP
5' 3'
3'
PB' gag' pol' env' U3' R' U5'
U3 R U5 PB
5'
3'
3'
PB' gag' pol' env' U3' R' U5'
U3 R U5 PB
5'
U3' R' U5' PB'
U3 R U5 PB gag pol env U3 R U5
5'
3'
3'
5'
LTR
LTR

(b) Synthesis of Plus-Strand DNA

For synthesis of plus strand DNA, HIV like other lentiviruses requires two primers; a polypurine track (PPT) which is a run of at least nine A and G residues and borders the U3 domain in the 3' LTR and a central PPT (cPPT) located at the end of the *pol* gene sequence. The PPT sequence avoids digestion by RNase H, and its 3' end primes plus-strand DNA synthesis. Following initiation plus strand DNA is elongated to the end of the minus strand DNA template to produce plus strand strong-stop DNA. A second strand transfer then occurs so the tRNA primer binding site in the plus strand viral DNA is base paired with the 3' end of the minus strand strong stop DNA. RT continues, elongating the plus strand DNA to the central termination signal (CTS) located immediately 3' to the cPPT. In addition, plus strand DNA initiated at the cPPT is elongated to the end of the minus strand DNA template. About 100 nucleotides of plus strand DNA, between the cPPT and the CTS, are displaced. Cellular enzymes remove the displaced sequences and seal the plus strand to yield double-stranded linear DNA which has an LTR at each end. The primer RNAs are removed from the 5' ends of each strand. The diploid genome is not necessary for reverse transcription and diploidy may provide a means for repairing physical damage to the genome by allowing recombination during reverse transcription.

(ii) Integration of Viral DNA

Integration is a process unique to retrovirus replication. Following viral DNA synthesis, the core structure containing linear DNA, the CA, the integrase enzyme (IN) and possibly RT and NC proteins, enters the nucleus. Evidence would suggest that nuclear localisation signals are present in the matrix protein and/or Vpr. Once in the nucleus HIV is integrated into the host genome in its linear form, flanked by both the 5' and 3' LTRs. The ends of the LTR contain *"att"* sites which provide a signal for integration which is catalysed by the viral IN enzyme. In order for integration to occur the 3' terminal two bases at either end of the viral DNA are removed by the cleavage reaction of the IN, leaving a 3'-OH end. The 3' ends of the viral DNA are joined to previously nicked sites in the host DNA. The integration reaction leaves single-stranded gaps and two mismatched nucleotides at each 5' terminus. Host cell enzymes remove the mismatched nucleotides, fill the gaps, and nick-seal the remaining ends.

(iii) Transcription

Once the provirus is integrated into cellular DNA, the next stages of replication occur utilising cellular systems. Cellular RNA polymerase II synthesises viral RNA from the proviral DNA, similiarly to cellular mRNA. The provirus is transcribed into a single RNA precursor, which is subsequently processed by polyadenylation at the 3' end of R to yield a genome-length molecule and by splicing to generate subgenomic mRNA species.

The LTRs are identical in nucleotide sequence but functionally different. Only the 5' LTR is active for transcription initiation and it is likely that the 3' LTR configuration is not open to transcription factors. The U3 domain of HIV contains basal promoter elements, including a TATAA box for initiation by host cell RNA polymerase II, and sites for binding the cellular transcription factor SP1. Immediately upstream of the core promoter and TATAA box, HIV contains one or more copies of the 10 bp sequence (GGGACTTTCC) that are recognised by the T-cell enhancement factor NF-kB. This may be significant in HIV infection as cell activation is required for HIV replication and T cells are generally quiescent.

(iv) Processing of Viral Transcripts

(a) Capping Group

A m^7GPPP capping group is attached to the 5' end of viral mRNAs during synthesis by the cellular transcription machinery. This structure may be important for translation.

(b) Internal Methylation

Retroviral genomes are posttranscriptionally modified by occasional methylation on the 6 position of A residues. It has been suggested that methylation is important for the regulation of splicing.

(c) Polyadenylation

Termination of transcription does not occur in the 5' LTR but only in the 3' LTR, perhaps due to differences in the secondary structures of the LTRs. Most primary transcripts are then processed by splicing and 3' -end maturation to yield mRNAs. Primary transcripts are endonucleolytically cleaved and then a poly(A) tail of 200 to 300 nucleotides is added by the poly(A) polymerase. Signals in U3 and R regions of the 3' LTR (AAUAAA and a downstream GU-rich element) are recognised by cellular functions which add poly-A tails to the 3' ends of viral transcripts. Poly(A) at the 3' end of mRNAs is implicated in stability, translatability, and in translocation from the nucleus to the cytoplasm.

(d) Splicing

Full-length HIV transcripts serve three roles: firstly as genomic RNA in progeny virions, second as mRNA for translation of Gag and Gag-Pol polyproteins in the cytoplasm and third as precursors for alternatively spliced mRNAs that are also translated in the cytoplasm to produce Env gp and accessory proteins. The ratio of spliced to unspliced viral mRNA is controlled by the regulator of viral gene expression Rev functioning through a Rev-responsive element (RRE) (For splicing see section 4.1 to 4.3). The transactivating protein Tat and the regulatory protein Rev are expressed from multiply spliced RNAs and accumulate early after infection.

Later as the regulatory proteins increase in amount, the amount of *gag-pol* and *env* mRNA would increase rapidly.

(v) Translation

The translation of retrovirus mRNA apparently follows a standard scanning model in which ribosomal subunits bind initially to the capping group and move along the RNA until the *gag* AUG initiation codon is encountered. A shift of reading frame must occur for read-through into the next coding region. There is apparently an occasional slippage of the ribosome to the -1 frame. As a consequence only 1/10 or 1/20 as much Pol as Gag is made. This ensures a proper ratio of proteins to one another and provides the *pro* and *pol* proteins in association with *gag* so they can be incorporated into virions.

Unlike the translation of *gag* and *pol,* which takes place on free polyribosomes using full length RNA; the *env* protein product, like other cell surface proteins, is synthesized on polyribosomes associated with the rough endoplasmic reticulum using spliced subgenomic RNA.

(vi) Virion assembly

The first event in virion assembly is the formation of nucleoprotein complexes composed of p55 Gag as well as the Gag-Pol polyprotein (p160), and genomic viral RNA. Cleavage of Gag-containing polyproteins during assembly is mediated by the viral protease to produce a mature nucleocapsid composed of fully processed Gag (MA, CA, NC, p6, P1, and P2) and Pol products (PR, RT, RNAse H, and IN) as well as two molecules of single-stranded RNA genome. Oligomers of Env gp are inserted into the plasma membrane, and the MA domain of p55 Gag is presumed to interact with the cytoplasmic tail of the transmembrane subunit in the Env oligomer during virion assembly. The viral nucleoprotein complex extrudes or buds through the plasma membrane to produce a virion with a nucleocapsid surrounded by a lipid bi-layer membrane which contains oligomers of Env gp. Selected host cell proteins are also incorporated into mature virus particles.

1.8 Anti-retroviral Therapy (reviewed in Skowron, 1996).

It is estimated that, in HIV-1 infection, about 10^{10} virions are produced daily and about 10^{9} CD4 cells are destroyed and produced daily (Ho *et al.*, 1995). An inhibitor of viral replication could prevent this cell loss, however several factors in the HIV replication cycle are themselves challenges. First integration means proviruses can remain latent or can be expressed at low levels. Second HIV infection occurs throughout the body, including lymph nodes and the central nervous system. Thus delivery of an antiviral agent to all target cells is difficult. Third, reverse transcription does not involve a proof reading reaction , therefore variant genomes including anti-viral resistant strains are readily generated.

The first treatment available was Zidovudine (ZDV/AZT), a nucleoside analogue reverse transcriptase inhibitor. Nucleoside analogues are structurally similiar to the natural dideoxynucleosides that the HIV RT incorporates into complementary DNA strands. Nucleoside analogues are incorporated into growing DNA strand and prevent subsequent elongation. Unfortunately it became clear that individuals treated with AZT only benefit transiently before resistant viral strains emerged. Multiple mutations in the RT gene were seen in 90% of late-stage and 30% of early-stage patients after 1 year of AZT therapy. More recently protease inhibitors have become available for the treatment of HIV infection. These antiviral agents bind sites on the HIV protease active site and suppress

Table 1.3 : Agents currently available or under study for HIV antiretroviral chemotherapy.

Mechanism of Action	Examples	Comments
Nucleoside analogue reverse transcriptase inhibitors	AZT, Zidovudine	Prolonged use associated with emergence resistant virus strains.
	ddI, didanosine	Combination therapy reduces the emergence ddI resistance.
	ddC, Zalcitabine	Most potent of the nucleoside analogues. ddC resistance is uncommon.
	d4T, stavudine	d4T generally well tolerated.
	3TC, Iamivudine	Rapid development resistance resulting from single point mutation in RT. This mutation restores sensitivity to AZT
Non-nucleoside analogue reverse transcriptase inhibitors	Nevirapine	Transient antiretroviral effect. Resistance.
	Delavirdine	Rapid development resistance.
Protease inhibitors	Saquinavir	Poor absorption. Low cross-resistance to other protease inhibitors.
	Ritonavir	Ritonavir-resistant mutant cross-resistant to indinavir.
	Indinavir	Reduction in viral load of 1.5 log at 6 months. Increases CD4 $100x10^6$/litre for more than 1 year.

HIV replication by inhibiting proteolysis of Gag-Pol polyproteins, rendering progeny virus non-infectious. Twelve or more antiviral agents may be available for use, alone or as components in double or triple therapy (table 1.3). These include nucleoside analogue and non-nucleoside reverse transcriptase inhibitors, and protease inhibitors.

The advantages of combination therapy in HIV infection include an additive or synergistic effect on antiviral activity and a delay or avoidance of drug resistance. In many cases after the use of a protease inhibitor in combination with AZT and 3TC, no virus was detectable in peripheral blood (Ho, 1997). However, from antigen and HIV antibody tests of PBMCs or plasma isolated from treated individuals it is not possible to speculate whether or not the virus has been cleared. It may be that virus is still detectable in the lymphoid tissue of these individuals and when therapy is ceased the viral load may recover.

Before therapy is prescribed for a particular individual it is necessary to first evaluate the risk of disease progression against possible adverse effects of therapy and therefore to assess risk factors for each drug. Once a course of treatment has been decided it is essential to monitor the response by following general health, the CD4 count, and the plasma viral load. It is also important that a course of therapy that is best tolerated by each individual is used, and that the emergence of resistant viral strains is monitored throughout treatment.

CHAPTER 2 : Materials and Methods

MATERIALS AND METHODS

2.1 Cell separation

(i) Blood samples

20 to 30 mls of whole blood were collected in ethylenediaminetetraacetic acid (EDTA) blood tubes (Sarstedt, Leicester, UK) from 34 HIV-1 seropositive individuals. The individuals were from two study groups, the Heterosexual Transmission Study (The City Hospital, Edinburgh) (table 2.1) and those attending Genito-Urinary Medicine (The Royal Infirmary, Edinburgh) (table 2.2). Immunological and virological information on disease progression was available for all but two individuals (tables 2.1 and 2.2). Risk factors for infection included intravenous drug use and sexual contact with an HIV-positive individual. Only five individuals were receiving or had received antiviral treatment in the previous six months.

CD4, CD8 and total lymphocyte counts were available for the samples studied and at monthly or two monthly intervals during the previous 12 months. Average CD4, CD8 and lymphocyte counts over a 6 month period previous to the study were calculated. Using cell counts from 12 months before the study, the percentage cell loss was calculated for CD4 and CD8 lymphocytes. CD4 counts ranged from less than one to more than 980 CD4 lymphocytes per μl. 21 of the patients had CD4 counts associated with late stage disease (CD4 count $<200/\mu l$),

Table 2.1 : Risk Group, Treatment, Cell Counts, and Blood Virus Load from the Heterosexual Transmission Study.

Patient	Risk Group	Therapy	AIDS D.I.	Mean lymp	CD4 Abs	Mean CD4	CD4 Loss (%)	CD8 Abs	Mean CD8	CD8 Loss (%)	Mono	p24 antigen (pg/ml)
P1	Het/IVD	n	A	1184	162	228	63	646	677	26	190	<8
P2	IVD	n	A	3146	959	938	-140	2204	1422	-92	550	112
P3	Het	n	PCP	630	31	80	92	336	524	66	284	<8
P4	IVD	n	A	2978	983	860	0.7	2102	1739	-8	320	33
P5	IVD	n	PCP	855	2	4	0	571	875	0	270	<8
P6	Het/IVD	n	A	853	234	236	11	361	433	45	160	<8
P7	Het/IVD	n	A	727	24	63	72	456	505	37	150	<8
P22	Homo	n	PCP	357	<1	0	0	235	348	1	120	87
P23	Het	n	A	N	385	305	16.3	764	764	11	530	<8
P24	Het	n	OC	978	7	14	53	372	552	-43	280	53
P25	Het	n	A	1771	638	576	-18	1298	1179	-32	520	<8
P26	Het/IVD	n	A	285	<1	0.5	97	70	105	74	160	64
P27	IVD	n	A	4309	35	54	62	1951	2291	31	420	<8
P28	IVD	n	A	1400	128	143	-2	774	692	-7	170	N
P29	Het	n	A	227	6	13	0	102	148	26	160	N
P30	IVD	n	A	1731	238	279	-8	1244	1430	-25	740	<8
P31	N	n	N	N	35	N	N	721	N	N	N	N

Het=heterosexual contact; IVD=intravenous drug use; Homo= male homosexual; n= no therapy in previous six months, AIDS D.I. = AIDS defining illness; PCP= *Pneumocystis carinii pneumonia:* OC= oesophageal candidosis; A= Asymptomatic.

CD4 , CD8, Monocyte counts (cells/µl). Mean CD4 and CD8 counts are an average of 3-6 results taken over a six month period before the sample was analysed. CD4 and CD8 loss are a % loss from the value recorded 12 months prior to the sample. N= Data not available.

Table 2.2 : Risk Group, Treatment, Cell Counts, and Blood Virus Load from the Edinburgh Genito-Urinary Medicine Cohort.

patient	risk group	Therapy	AIDS D.I.[i]	Mean lymp[n]	CD4 Abs[m]	Mean CD4[n]	CD4 Loss[o]	CD8 Abs[m]	Mean CD8[n]	CD8 Loss[o]	mono[m]	p24[q]
S04	IVD[b]	n[d]	A[l]	693	184	73	-167	945	438	-74	380	24
S05	IVD	n	A	899	323	406	33	301	537	60	160	<8
S06	IVD	n	A	680	56	56	-20	458	451	-54	N	<8
S07	N	n	A	970	20	22	56	1013	1058	56	N	N
S08	N	n	A	1397	6	8.3	33	896	948	7	220	N
S09	N	n	A	578	11	3	N	426	411	-28	N	N
S11	Het[a]	n	PCP[j]	1656	74	85	33	1151	1227	31	240	8
S14	Homo[c]	n	A	1843	382	354	20	1431	1136	14	510	74
S15	Homo	AZT[e]/DDI[f]	A	762	7	10	93	521	509	42	1040	50
S19	Homo	DDC[g]	A	840	52	90	54	427	580	30	250	45
S21	Homo	n	A	911	293	386	40	843	811	15	490	<8
S22	Homo	n	A	3025	366	418	21	2732	2184	-103	590	>125
S24	N	3TC[h]/AZT	N[p]	489	34	23	32	461	330	-52	280	N
S25	Homo	AZT/DDI	A	2437	400	420	N	1573	1573	N	230	N
S26	Homo	AZT/3TC	N	1613	187	184	50	1173	954	-44	210	N

a Het=heterosexual contact; b IVD=intravenous drug use; c Homo= male homosexual; d n= no therapy in previous six months; e AZT= Azidothymidine; f DDI= Dideoxyinosine; g DDC= Dideoxycytidine; h 3TC= Lamivudine.; i AIDS D.I. = AIDS defining illness; j PCP= *Pneumocystis carinii pneumonia;k* OC= oesophageal candidosis; l A= Asymptomatic.

m CD4 , CD8, Monocyte counts (cells/µl); n Mean CD4 and CD8 counts are an average of 3-6 results taken over a six month period before the sample was analysed; o CD4 and CD8 loss are a % loss from the value recorded 12 months prior to the sample; p N= Data not available; q p24 antigen (pg/ml).

whereas the other 11 had no symptoms and a CD4 count of more than 200/μl. Of the individuals with CD4 count less than 200, 5 had an AIDS defining illness. Absolute CD8 counts ranged from 70 to 2732/μl and monocytes from 120 to 740/μl. p24 antigen levels were measured in 24 samples using a commercial kit (Coulter, Miami, USA) according to the manufacturers instructions.

(ii) Determination of individual absolute cell counts.

Absolute cell counts were determined by flow cytometry in the HIV Immunology laboratory, The Royal Infirmary Edinburgh. An Ortho Trio patient summary report (Ortho-Clinical Diagnostics, Herts, UK) was produced illustrating the CD3-positive T cells, CD4-positive T cells, CD8-positive T cells, CD19-positive B cells, CD16-positive, CD3-negative natural killer cells as cells per microlitre of whole blood. A further study involved 40 HIV-1 positive individuals. The number of CD4 and CD8 memory and naive cells were determined using a panel of antibodies against CD4, CD8, CD45RA (naive cells), and CD45RO (memory cells). (FACS analysis carried out by Helen Mason).

(iii) Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Blood samples were diluted with an equal volume of phosphate buffered saline (PBS) (GIBCO BRL, Paisley, UK) and slowly layered over a ficoll-hypaque density gradient, Lymphoprep (Nycomed, Oslo, Norway). 3 mls of lymphoprep

were used for every 10 mls of diluted whole blood. Samples were then spun for 30 minutes at 2100 rpm (500xg) with no brake. PBMCs and platelets collected on top of the gradient because they had a lower density and red blood cells, which have a higher density pelleted below the gradient. The top layer of plasma was removed, aliquoted and stored in liquid nitrogen. The PBMC layer was removed and washed twice with PBS for 10 minutes at 1000 rpm (100xg) removing any ficoll-hypaque and platelets. Viable cells were counted by means of a haemocytometer, with trypan blue exclusion. 2 aliquots of $2x10^5$ PBMCs were pelleted and frozen in liquid nitrogen for DNA and RNA extractions. The remaining PBMCs were resuspended in PBS for subsequent cell separations.

For each HIV-positive cell separation, negative PBMCs supplied by the Edinburgh and South East Scotland Regional Transfusion Centre (RTC) were run in parallel as negative controls. It was essential to show that none of the cell separation techniques used interfered with the polymerase chain reactions (PCR) carried out later.

(iv) Isolation of T Cells Using Rosetting Procedure.

For 17 samples, a rosetting procedure was used to isolate T and non-T cells from PBMCs. 20 mls of sheep red blood cells (SRBCs) in Alsevers solution (Scottish Antibody Production Unit [SAPU], Lanark, UK) were washed twice at 2100 rpm (500xg) for 10 minutes in PBS. 1 ml of the SRBC pellet was incubated with 1 unit of neuraminidase (Sigma, Dorset, UK) in PBS for 1 hour at 37°C. The

SRBCS were washed twice with PBS then resuspended in 49 mls of RPMI (GIBCOBRL, Paisley, UK).

No more than $2x10^7$ patient PBMCs were washed and resuspended to a final volume of $1x10^7$ PBMCs/ml in PBS For every ml of PBMCs 2 mls of FCS and neuraminidase-treated -SRBCs were added and the mixture was incubated at 37°C for 10 minutes. The cells were then loosely pelleted by spinning at 800 rpm (70xg) for 5 minutes and the pellet was incubated for 1 hour on ice.

The SRBC, FCS and PBMC mixture was resuspended and layered over ficoll-hypaque as previously described . The top layer of cells, non-T cells were washed twice, counted and used for further cell separations. The SRBCs bound the CD2 receptor found on T cells, forming rosettes, which formed a loose pellet below the ficoll-hypaque. After removing the gradient, the SRBCs were hypotonically lysed and the T cell fraction was washed twice with RPMI and resuspended in PBS for further separations.

(v) Magnetic separation of E-rosette positive and negative cells.

CD3, CD4, CD8, CD14 and CD19 monoclonal antibodies were supplied by SAPU. The CD21 monoclonal was obtained from The Binding Site, Birmingham, UK, HLA-DR from The American Tissue Culture Collection, Maryland, USA, and CD11b was donated by Peter Beverly, University College, London, UK.

100 µl (1 mg) of magnetic beads coated with antibody to mouse immunoglobulin (IgG) (Immunotech, Marseilles, France) were incubated with 5 µg

of each mouse monoclonal antibody. The beads and antibody were annealed overnight in a small volume (50 µl) at 4°C and then washed twice with PBS to remove any unbound antibody.

T cells and non-T cells were serially incubated with these antibody coated beads for 30 minutes and the cells which bound to the beads were removed using a neodymium-iron-boron magnet (Dynal). The E -rosette positive fraction was incubated first with anti-CD4 to select T helper cells and then with anti-CD8 to select CD8 lymphocytes. Non-T cells or the E- rosette negative fraction were incubated with anti-CD3 to remove any contaminating T cells, and then monocytes were isolated by means of ant-CD11b and anti-CD14 beads. B cells were depleted with anti-CD19 and 21, from the E-rosette negative monocyte depleted fraction. Dendritic cells were isolated from the fraction depleted of T cells, monocytes and B cells using anti-CD4 and HLA-DR. Samples of each depleted fraction were taken for flow cytometer analysis. Unfortunately these beads were too large for the flow cytometer so the positively selected cells could not be directly analysed. Viable cell counts for positively selected cells were calculated by counting depleted cells at each selection step.

(vi) Magnetic separation using the miniMACS (monoclonal antibody cell sorter)

For 15 of the isolated PBMCs an alternative cell separation procedure was used. Direct MACS (monoclonal antibody cell sorter) microbeads (Miltenyi Biotec Ltd, Camberly, UK) conjugated to antibodies directed against CD14, CD4, CD8, and CD56 were used. MACS beads are only 50 nm in diameter and neither change the scatter properties of the cells in the flow cytometer nor do they influence the light microscopic appearance of the cell. Therefore positively selected cells could be directly counted and analysed by flow cytometry using this method.

A miniMACS magnet was used allowing the separation of a few hundred to $2x10^8$ cells. PBMCs were resuspended carefully to ensure a single cell suspension in 180 µl of PBS. Then 40 µl of each directly conjugated magnetic beads were added sequentially and the cells were incubated for 15 minutes at 6-12°C. A separation column was placed in the miniMACS magnet and washed by running through 500 µl of PBS. The magnetically labelled cells were applied to the column in a 500 µl volume. The column was then rinsed three times with 500 µl of PBS. The effluent was collected as a negative fraction. The negative fraction was spun at 100 rpm (100xg) for 5 minutes to pellet the cells and the whole procedure was repeated for the next antibody. For the positive cell fraction the separation column was removed from the magnet and the cells flushed out using 1 ml of PBS and a plunger supplied with the column. Aliquots of each cell fraction were used for flow

cytometry in order to monitor the cell purity and viable cell counts were made at each step.

The PBMCs were incubated twice with anti-CD14 to remove all the monocytes. Then they were incubated with anti-CD4 to isolate CD4 T lymphocytes, dendritic cells, and T cells expressing CD4 and CD8. Natural killer cells were then removed using anti-CD56 and finally CD8 lymphocytes were selected using CD8 coated MACS beads.

(vii) Isolation of CD4 and CD8 positive lymphocytes using Dynabeads

Four samples, two HIV-positive and two negative controls were separated using magnetic Dynabeads (DYNAL UK Ltd,Wirral, UK) conjugated with anti-CD4 and anti-CD8. Monocytes were first depleted by plastic adherence. Briefly PBMCs were incubated overnight at 37°C in a plastic tissue culture flask with Iscoves medium (GIBCOBRL, Paisley, UK). Any monocytes adhere to the plastic surface.

Anti-CD4 and CD8 Dynabeads were washed twice with PBS using a magnet then resuspended to a final bead concentration of $2x10^7$ per ml. Beads were added to the monocyte depleted cell fraction to give a final concentration of at least $1x10^7$ dynabeads/ml. The cells were incubated for 20 minutes at 4°C with gentle rotating and the selected cells removed by means of a magnet. The cells were first incubated with anti-CD4 beads then the depleted fraction was treated with anti-CD8 beads.

(viii) Storage of selected cell subsets.

For every sample 1 ml aliquots of plasma were stored in liquid nitrogen. Each cell fraction was stored in three aliquots. Two were snap frozen as cell pellets in liquid nitrogen for RNA and DNA extractions. The other aliquot was resuspended in 0.5 mls of freezing medium containing 50% RPMI, 40% foetal calf serum (FCS), and 10% DMSO (dimethyl sulphoxide). This aliquot was frozen slowly and then stored in liquid nitrogen so these slow frozen cells could be used in cell culture.

(ix) Purity of isolated cell subsets.

The process of isolating subsets of mononuclear cells by antibody-coated magnetic beads (Immunotech) prevented analysis of the selected cells by fluorescence-activated cell sorting (FACS) techniques. However, the purity of cell fractions could be inferred by measurement of the frequencies of different cell types in the residual cells after removal of a particular fraction. The small size of MACS microbeads allowed direct staining of the magnetically labelled cells with fluorochrome conjugated antibodies and subsequent analysis by flow cytometry.

Cell populations were stained with mouse monoclonal antibodies conjugated to fluorescein isothiocyanate or phycoerythrin for FACS (Becton-Dickinson, Cowley,UK). Cells were stained with a panel of monoclonal antibodies including, anti-CD3, CD4, CD8, CD14, CD16, CD19, CD21, and HLA-DR. Cells were incubated with 10 µl of each conjugated antibody for 30 minutes on ice, in the dark.

Any unbound antibody was removed by washing twice with PBS. The cells were resuspended in FACS fix (2% paraformaldehyde) then analysed by flow cytometry. Unstained control cells and positive control cells were run in parallel with each sample. Flow cytometry was carried out under the supervision of Donald Innis, Kings Buildings, Edinburgh.

2.2 Extraction of nucleic acid

Nucleic acid was isolated from cell pellets and 100 µl plasma by incubation in TNE buffer [0.11 M sodium chloride, 55 mM Tris pH8, 0.55% sodium dodecyl sulphate (SDS)](BDH Laboratory Supplies, Poole, UK) and 1 mg/µl proteinase K (Boehringer Mannheim, Sussex, UK) for 30 minutes at 37°C in the presence of poly A (Boehringer Mannheim) precipitant (40 µgmµl) in a total volume of 500 µl. 450 µl of water saturated phenol (Rathburn Biochemicals, Walkerburn,UK) was added and the samples shaken vigorously for 5 minutes. After centrifugation at 15000 rpm (14000xg) (Heraeus benchtop centrifuge) for 5 minutes the aqueous layer was transferred to 450 µl Chloroform/iso-amyl alcohol (50:1) (BDH Laboratory Supplies) and placed on a shaker for 2 minutes. The centrifugation step was repeated and the aqueous layer transferred to a fresh eppendorf containing 40 µl sodium acetate (pH 5.2) (Sigma) and 800 µl ethanol (-20°C) (Rathburn Biochemicals). After thorough mixing the nucleic acid was precipitated overnight at -20°C. The nucleic acid was collected by centrifugation at 15000 rpm (14000xg)

at 0°C. The pellet was washed twice with 80% ethanol and resuspended in 25 µl nuclease free water.

2.3 Quantification of HIV proviral sequences.

Proviral sequences were quantified using a previously described limiting dilution and nested PCR approach (Simmonds *et al.*, 1990b). The double PCR was performed as two sequential PCR reactions. The initial reaction contained the DNA to be amplified together with a sense and antisense primer spanning the V3 and gag regions. The nucleotide sequences of the primers and the position of the 5' base in the HXB2 genome (Myers *et al.*, 1991) are given in table 2.3.

Table 2.3: Primer sequences

Primer	Sequence (5'-3')	Position of 5' base
V3a	tacaatgtacacatggaatt	6957
V3b	tggcagtctagcagaagaag	7009
V3c	ctgggtcccctcctgagg	7331
V3d	attacagtagaaaaattcccc	7381
*gag*e	gcgagagcgtcagtattaagcgg	795
*gag*f	gggaaaaaattcggttaaggcc	835
*gag*g	cttctactactttacccatgc	1248
*gag*h	tctgataatgctgaaaacatggg	1296

V3 primers provided equivalent quantitation to primers in the gag region (Donaldson *et al.,* 1994a). This reaction was carried out in a 50 µl volume of PCR buffer containing 0.4 units DNA *Taq* polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl pH 9.0, Triton X-100, 1.5 mM $MgCl_2$, 30µM each of dGTP, dATP, dTTP,dCTP and 0.25µM of each of the outer nested primers. PCR was performed on serial ten fold dilutions of DNA over 25 heat cycles.

The reaction conditions were, denaturation 94°C for 40 seconds, primer annealing 50°C for 50 seconds and an extension step at 72°C for 90 seconds. Following initial amplification with outer primer pairs, 1 µl of the product was transferred to a second tube containing 20 µl of reaction buffer, *Taq* polymerase (0.015 U/µl) and a pair of primers lying within one of the outer primer pairs used in the first reaction. Product DNA was amplified for a further 25 heat cycles using the same program as before. The product of the second PCR reaction was visualised by electrophoresis on a 1% agarose gel (Sigma) and by ultra-violet transillumination in the presence of ethidium bromide.

After preliminary quantifications using serial ten fold dilutions of DNA and subsequently (using V3 primers), on two fold dilutions in quadruplicate around the end-point, between 10 and 20 replicates were then carried out at the end-point in order to more accurately determine the virus load.

In addition to the selected subsets (CD4 T cells, CD8 T cells, B cells, monocytes and dendritic cells) PCR quantitation was also used to determine the

provirus load in PBMCs and the residual E-rosette positive populations after CD4 and CD8 cell depletions of seven patients. Residual cells from this fraction could be unselected cytotoxic T cells (CTLs) or T helper cells in which CD4 was down-regulated by infection with HIV. Quantification results were expressed as proviral copies per 10^6 cells. All separations, extractions and amplifications were carried out with parallel samples of PBMCs isolated from buffy coats derived from HIV negative blood to serve as negative controls.

2.4 Analysis of quantification results.

The relative frequencies of CD4 T lymphocytes, CD8 lymphocytes, monocytes and B cells in the original mononuclear cell populations were estimated by FACS analysis. Numbers of dendritic cells were estimated by cell counting before and after depletion of the E-rosette negative population by anti-CD4 and HLA-DR coated magnetic beads. To estimate the numbers of cells used for PCR cell counts taken at each depletion with specific monoclonal antibodies were used. The only exception were samples from individuals with CD4 counts less than 200 per µl of whole blood, where the low level of cells lost during magnetic separation greatly exceeded the numbers of selected cells. For these samples we therefore used the absolute CD4 counts derived from FACS analysis of the original blood samples to estimate cell numbers.

For each cell subset a final PCR reaction was carried out with 20 replicates at the determined end-point. Using the volume of extracted nucleic acid used in the

end-point PCR reaction and the number of cells extracted, the number of provirus copies per $1x10^6$ cells was estimated. In order to determine the actual number of provirus copies/10^6 cells we used the Poisson distribution to calculate the frequency of target sequences per replicate, using the following formula: $f = -\log(1-p)/d$ (where f = the frequency of infection, p = proportion of positive samples and d = dilution). To ensure that all proviral sequences were accounted for by this method of analysis, we compared the total number of provirus-bearing cells from unfractionated PBMCs with the sum of each of the component subsets analysed. For example, the frequency of provirus in PBMCs from p26 was 8 copies/10^6 cells. In this particular case, the contribution to the proviral sequences in the CD8 cells was 58 copies per million CD8 cells, multiplied by their frequency in the total PBMC population (70 000 CD8 cells/ml in a total mononuclear cell concentration of 600 000/ml: 11.6%). Therefore CD8 cells contribute 6.8 proviral copies/ml to the mononuclear cell virus load. The contributions of CD4 lymphocytes (2.1 copies/10^6 mononuclear cells), from monocytes (1.1 copies/10^6 mononuclear cells) and DCs (1.3/10^6 mononuclear cells) were calculated similarly. The total of these individual contributions was 11.3 copies /10^6 cells, close to the virus load measured by PCR on unfractionated mononuclear cells (8 copies/10^6 cells). These observations independently validated the methods used for quantitation of cells and provirus. This analysis was carried out on a total of seven patients and yielded a total PBMC proviral load/total of the subsets load ratio of between 0.4 to 1.56. These values are

consistent with the accuracy of the methods used for cell counting and quantification of HIV sequences.

In order to illustrate that virus detected in a cell subset was not due to contamination of the cell fraction with other cell types, the levels of contaminating cells were determined by FACS analysis. These values could then be used in parallel with the PCR results to show that for example CD4 contamination of the CD8 fraction could not account for the detected provirus.

2.5 DNA sequencing of PCR product

(i) Biotinylated PCR for solid-phase sequencing

For patients p2, p5 S4, S6, and S25, single molecules of HIV DNA obtained by limiting dilution PCR were used for sequencing the V3 region of the envelope gene. It was necessary to sequence single molecules as the heterogeneity of the *env* gene means that a consensus sequence would be impossible to interpret. For positive reactions the secondary reaction was repeated in a 100 µl volume using the inner sense primer and a biotinylated anti-sense primer.

(ii) Dynabeads for solid-phase sequencing.

Streptavidin coated Dynabeads (Dynal) were used to isolate biotin-labelled target molecules. The Dynabeads were supplied as a suspension of 6.7×10^8 beads per ml (10 mg/ml) in PBS pH 7.4. 20 µl of beads were removed per biotinylated

PCR product and washed twice with BW (10 mm Tris HCL pH 7.5, 1 mM EDTA, 2.0M NaCl) (BDH Laboratory Supplies) using magnetic apparatus. The beads were then resuspended in 40 µl of BW per reaction and incubated for 20 minutes at room temperature with 40 µl of biotinylated PCR product. After washing twice with BW the DNA was denatured with a 10 minute incubation in 0.15 M sodium hydroxide (NaOH) (Sigma) leaving only the biotinylated strand bound to the beads. The beads were then washed with 0.15 M NaOH, BW and TE (Tris HCl pH 7.5, 1mM EDTA) and were resuspended in 20 µl TE.

(iii) Solid-phase sequencing

Nucleotide sequencing was carried out using the Sequenase-2 kit (United States Biochemicals) with [^{35}S] dATP (Amersham, Buckinghamshire, UK) according to the instructions of the manufacturer. Briefly, 0.5 pmol of primer, 2 µl 5X sequenase reaction buffer and 5 µl DNA bound to Dynabeads were incubated at 65°C for 5 minutes then allowed to cool slowly to room temperature. 5 µl of this annealing mix was then added to an extension mix containing 0.1 µl labelling mix, 1 µl 0.1 M dithiothreitol (DTT), 3.25 units Sequenase enzyme and 5 µCi [^{35}S] dATP. 3 µl of the extended sequencing reaction were added to 2.5 µl of termination mix for each of the nucleotides G, A, T, and C. 4 µl of stop solution (95% formamide, 20 mm EDTA, 0.05% Bromophenol blue, and 0.05% xylene cyanol FF) were added to each of the termination mixes.

(iv) Acrylamide gel electrophoresis

The sequencing reactions were run on a 6% acrylamide gel. The gel mix contained 21 g urea (BAH Laboratory Supplies)), 6 mls of long ranger liquid acrylamide/Bisacrylamide (FMC Bioproducts, Rockland USA), 5 mls 10x TBE (0.089 M Tris, 0.089 M Boric acid, 0.02 M EDTA), 0.05 g ammonium persulphate (Sigma) and 20 µl TeMed (Sigma). The gel mix was then dissolved in a final volume of 50 mls with distilled water.

Glass plates were cleaned with 70% (v/v) ethanol then acetone. The plates were then assembled with flat spacers. The gel was poured taking care to avoid bubbles and left to polymerise. The gel was pre-run at 75 volts for 10 minutes using 1x TBE as the electrophoresis buffer. The samples were heated to 95°C for 7 minutes then loaded on the gel. The gel was dried and exposed to X-ray film for 1-3 days. No fix step was required with the brand of arylamine used.

(v) Analysis of sequencing gels.

Sequencing gels were read and aligned using a sequencing package developed in the department (Simmonic Software). Sequences were then analysed using the package MEGA (Molecular Evolutionary Genetics Analysis). Phylogenetic trees were constructed to compare patient cell subset sequences. A rooted tree was constructed with the HIV HXB2 sequence as an outgroup and the Jukes Cantor method to account for multiple substitutions. The bootstrap re-

sampling method (100 replicates) was used to assess the confidence of each node in the tree constructed.

2.6 Extraction of RNA

(i) Creating a Ribonuclease-Free environment

In order to prevent RNase contamination proper microbiological sterile technique was used and gloves were changed regularly. Reagents and utensils were set aside specifically for RNA work. Sterile disposable plastic ware was used which did not require pretreatment to inactivate RNases. Solutions used were treated with 0.05% diethyl pyrocarbonate (DEPC) overnight at room temperature and were autoclaved to remove any residual DEPC. The laboratory area used was first treated with RNase away (Molecular Bio-Products Inc, San Diego, USA)). All the reaction steps and reagents were stored or carried out on ice.

(ii) Stratagene RNA isolation kit.

RNA was extracted using the Stratagene RNA isolation kit according to the manufacturers instructions. Lysis solution A (Table 2.4) was prepared and the extraction protocol followed using the volume of reagents described in table 2.5 depending on the number of cells.

The cells to be extracted were centrifuged at 1000 rpm (100xg) for 5 mins and the supernatant removed. 100 μl of lysis solution and the correct volume of mercaptoethanol (β-ME) was added for every $1x10^6$ cells processed. A volume of

2 M sodium acetate (NaOAc) (pH 4.0), water saturated phenol, and chloroform:isoamyl alcohol were added and the mixture was and vortexed. The tube was mirofuged for 5 mins at 15000 rpm (14000xg) so two phases were clearly visible. The upper phase was transferred making sure not to take any of the interface layer, to a sterile RNAse-free microcentrifuge tube. The lower level containing phenol, proteins and DNA was discarded.

Table 2.4 : Lysis solution for RNA extraction

Total volume	50 mls	25 mls	10 mls	5 mls
Guanidinium Thiocynate (Sigma g-6639)	23.64 g	11.82g	4.8g	2.364g
1mM Na Citrate (pH 7)	1.25 mls	0.625 mls	0.25 mls	0.125 mls
Sarcosyl (Sigma L-5125)	0.5 g	0.25 g	0.1 g	0.05 g

Table 2.5: Volume of reagents used in RNA extraction protocol(μl)

Cell Amounts	$1x10^6$	$2x10^6$	$3x10^6$	$4x10^6$	$5x10^6$
Solution A	100	200	300	400	500
β-ME	0.72	1.44	2.16	2.88	3.6
2M NaOAC	10	20	30	40	50
Phenol	100	200	300	400	500
Chloro:Iso	20	40	60	80	100
Isopropanol	100	200	300	400	500
75% Wash	200	400	600	800	1000
DEPC H_2O	25	50	75	100	125

If less than 1 x 10^6 cells were used the procedure for 1 x 10^6 cells was followed and the RNA was precipitated with 1 μl glycogen. A volume of isopropanol was added to the RNA solution and mixed well. The sample was microfuged at 15000 rpm (14000xg) for 30 minutes after 1 hr at -70°C. The supernatant was removed and the pellet washed with 75% ethanol:25% DEPC-treated water, and the pellet dried. The pellet was resuspended in 20μl DEPC treated water containing 1 unit of RNasin (Promega) and stored in liquid nitrogen.

(iii) Isolation of mRNA

As an alterative to total RNA extraction, mRNA was extracted with the Mini-Message Maker (R and D Systems, Abingdon, UK) according to the manufacturers instructions. Briefly, 1 ml of the supplied lysis buffer was added to the cell pellets (no more than 10^7 cells) and DNA was sheared by passing the cells through a fine pipette tip. The cells were then microfuged at 15000 rpm (14000xg) for 3 minutes and the supernatant was transferred to a fresh tube. The supernatant was made to 1.5 mls with lysis buffer and 50 µl of oligo-dT latex beads were added. The tube was inverted ten times, incubated for 10 minutes at room temperature, and spun at 15000 rpm (14000xg) for 5 minutes. The pellet was resuspended in 350 µl of lysis buffer and transferred to a spin column. The spin column was spun at 15000 rpm (14000xg) for 2 minutes and the eluate discarded. The mRNA was eluted with 100 µl elution buffer at 70°C by spinning at 15000 rpm (14000xg).

(iv) DNase treatment

Purified RNA was treated with RQ1-RNase free DNase (Promega) in order to degrade any contaminating DNA. The RNA pellet was resuspended in 0.4 mls of DNase buffer (0.005M $MgCl_2$, 0.01M Tris pH 7.4) and 5 µl of DNase (50 U) were added. The RNA was incubated at 37°C for 30 minutes then at 97°C for 10 minutes to inactivate the DNase enzyme.

2.7 Detection of *env* messenger RNA.

(i) cDNA synthesis

cDNA was synthesised using 0.5 units AMV (Avian Myeloblastosis Virus) reverse transcriptase enzyme (Promega), 1x RT buffer (50 mm Tris HCl pH 8.3, 50 mM $MgCl_2$, 5mM DTT,5mM EDTA, 50µg/µl bovine serum albumin, 50 mm KCl), 0.8 mM dNTPs, 10% DMSO, 0.75 units RNasin, 0.1µM outer antisense primer and 5 µl of RNA in a final volume of 20 µl. The reaction mix was incubated at 42°C for 1 hour and stored at -70°C. Mock RT reactions were also prepared in order to determine whether DNA contamination was present.

(ii) Primary PCR Amplification.

Reaction mix was prepared as described in table 2.6 in a total volume of 50 µl.

Table 2.6 : mRNA PCR reaction mix.

Component	Stock concentration	Final concentration	Volume (µl)
ddH_2O			36.75
Taq DNA pol buffer	10X	1X	5
dNTPs	3 mM	0.03 mM	0.5
s5326 (antisense primer)	10 µM	0.25 µM	1.25
NARS (sense primer)	10 µM	0.25 µM	1.25
cDNA	1X	0.1X	5
Taq DNA pol (Promega)	10 U/µl	0.05 U/µl	0.25

The PCR heat cycle was as follows; 94°Cx5min for 1 cycle, 58°Cx5min for 1 cycle, 72°Cx1.5min, 94°Cx35sec and 58°Cx40sec for 30 cycles, followed by 72°Cx10min and 22°C for 1 cycle of 30 minutes.

Primers were selected so the sense primer was complementary to a sequence in the LTR region and the antisense was complementary to the *env* region (see figures 4.1 and 4.2). Only the spliced products *vif, vpr, tat* exon 1, and *vpu-env* were detected using the primers listed below:

NARS 5' GAGATCGTCACCGCGGGCTTGTCCC 3' (position 627 HXB2)

S5236 5' CCCATAATAGACTGTGACCCA 3' (position 6327 HXB2)

Half the volume of the amplified product was loaded a 1.5% metaphor gel.

(iii) Secondary PCR Amplification.

Reaction conditions were the same as those for the primary reaction. 0.1 μl of primary product was transferred to a thin walled tube and reaction buffer added in a final volume of 20 μl.

The following inner primers were used:

T5480 5' TGAGCCGAACGACTTCGC 3' (position 678 HXB2)

S5237 5' TCCCAAGGAGCAWGRTGCCCC 3' (position 6273 HXB2)

The amplified product was loaded on a 1.5% metaphor gel. The expected size of the products were; *vif* 1424 base pairs (bp) , *vpr* 905 bp, *tat* exon 1 514 bp, and *vpu-env* 314 bp.

(iv) Quantitative RT-PCR

RT-PCR only provides an answer as to whether or not a transcript is being expressed. Relative quantitation was carried out by running an actin message RT-PCR in parallel with the HIV *env* PCR. Every cell should express actin message so this PCR acted as an internal control to monitor RNA loss and RT efficiency between different reactions.

2.8 Detection of actin mRNA by RT-PCR

(i) cDNA synthesis

mRNA was annealed to an oligo (dT)15 primer and cDNA synthesis as described for *env* mRNA.

(ii) PCR Amplification.

5 µl of RNA was added to reaction mix as described in table 2.7.

Table 2.7 : Reaction mix for actin PCR.

Component	Stock concentration	Final concentration	Volume (µl)
ddH_2O			14.9
Taq DNA pol buffer	10X	1X	2
dNTPs	3mM	0.03mM	0.2
antisense primer and sense primer (Stratagene)	*25u*M	1*u*M	0.8
cDNA	1X	0.1X	2
Taq DNA pol (Promega)	10U/µl	0.05U/µl	0.1

PCR reaction conditions were as for *env* mRNA. 1 µl of primary product was transferred to a fresh tube and the PCR reaction repeated using secondary primers. The primers used were as follows:

Primary PCR Reaction

β-Actin-sense 5' TGACGGGGTCACCCACACTGTGCCCATCTA 3'

β-Actin-antisense 5' CTAGAAGCATTTGCGGTGGACGATGGAGGG 3'

Secondary PCR Reaction

β-Actin-sense 2 5' GCCCTGGACTTCGAGCAAGAGATGGCCAC 3'

β-Actin-antisense 5' CTAGAAGCATTTGCGGTGGACGATGGAGGG 3'

2.9 Detection of RNA by Access RT-PCR

The Access RT-PCR system (Promega) is designed for RT and PCR amplification in one reaction step. The reaction mix was prepared on ice: 1X AMV/T*fi* (*Thermus flavus*) buffer, 0.2 mM each dNTP, 1 mM outer sense and anti-sense primers, 1 mM $MgSO_4$, 0.1 U/μl AMV-RT, 0.1 U/μl T*fi* DNA polymerase, 10 μl RNA and to a final volume of 50 μl with nuclease free water. T*fi* polymerase is a thermostable DNA polymerase from *Thermus flavus*. Also supplied were positive control RNA, primers and a negative control. The reaction conditions were as follows (table 2.8):

Table 2.8 : Reaction conditions for Access RT-PCR

Reverse Transcription	1 Cycle	48°C	45 minutes
RT Inactivation and Denaturation	1 Cycle	94°C	2 minutes
Denaturation		94°C	30 seconds
Primer Annealing	40 Cycles	56°C	1 minute
Extension		70°C	2 minutes
Final extension	1 Cycle	70°C	7 minutes
Soak	1 Cycle	4°C	10 minutes

1 µl of primary PCR product was used in a secondary reaction and the product visualised using a 2% agarose gel stained with ethidium bromide.

2.10 Cell Culture

(i) Cell Medium

Medium was prepared in a sterile still air cabinet. Sterile plastic pipettes and autoclaved tips were used. All plastic cell culture flasks were sterile. For most cell culture complete RPMI was prepared as follows: 100 mls RPMI 1640 (GIBCO BRL), 1% penicillin (GIBCO BRL), 1% streptomycin (GIBCO BRL), L-glutamine 1% (GIBCO BRL), 100 µl interleukin-2 (IL-2) (MRC), 10 mls heat inactivated foetal calf serum (GIBCO BRL) and 30 µl phytohaemaglutinin (Murex, Dartford, England). Cell conditioned medium was also prepared by removing the supernatant from cultured cells and spinning at 2000 rpm (500xg) to remove any cell debris.

(ii) PBMCs

PBMCs were isolated by ficoll-hypaque centrifugation (See section 2.1) from a whole uninfected blood pack supplied by the Edinburgh and South East Scotland RTC. The washed cell pellet was resuspended in complete RPMI (1ml/10^6 cells). After incubation at 37°C for 72 hours in a plastic cell culture flask, the cells were split and more medium added when required.

(iii) CD4 Lymphocytes

CD4 lymphocytes were isolated using miniMACS beads (see section 2.1) as described and were cultured as for PBMCs. The cells were FACs analysed before and after culture.

(iv) CD8 Lymphocytes

CD8 lymphocytes were isolated with miniMACS beads as described (section 2.1) and were suspended in cell conditioned medium ($1x10^6$ cells/ml). The cells were then treated as for PBMC culture. The cells were analysed before and after culture.

(v) Dendriric cells

Dendritic cells were isolated after E-rosetting as described (section 2.1) and were resuspended in complete iscoves medium (GIBCO BRL) in place of RPMI. The cells were cultured as described for PBMCs and were analysed by light microscopy in order to detect any characteristic dendritic cell morphology.

CHAPTER 3 : The Distribution of HIV-1 Within Peripheral Blood Cell *in vivo*.

INTRODUCTION

3.1 The CD4 Receptor

The cluster determinant (CD4) molecule is a T-cell co-receptor that increases the affinity of a helper T cell for an antigen presenting cell through its interactions with major histocompatibility (MHC) class II molecules. CD4 has also been implicated in signal transduction pathways essential for T-cell activation. Many studies indicate that infection of target cells with human immunodeficiency virus type-1 (HIV-1) is dependent on surface presentation of CD4, which serves as specific virus receptor (reviewed in Levy, 1993; Bour *et al.*, 1995; Bachelder *et al.*, 1996). Early reports that HIV-1 infection *in vivo* was restricted to the CD4 expressing subset of T lymphocytes were followed by studies showing that anti-CD4 monoclonal antibodies (mAbs) could block both infection of CD4-positive target cells and subsequent formation of syncytia. CD4 serves as a receptor for HIV-1, HIV-2 and simian immunodeficiency viruses (SIV).

The CD4 molecule is a transmembrane glycoprotein of 58 kDa and consists of an extracellular region of 370 amino acids, a transmembrane region of 25 amino acids, and a cytoplasmic tail of 38 amino acids at the C terminal end. The extracellular portion of CD4 is folded into 4 distinct domains (D1-D4). The N-terminal D1 domain shares extensive structural and sequence homology with the variable region of immunoglobulin (Ig) light chains, and is composed of three hypervariable regions called complementarity-determining regions (CDRs). The other three domains are less closely related to Ig molecules at the level of primary

structure but fold similarly to Ig family domains, confirming that CD4 is a member of the Ig-like superfamily.

The HIV-1 envelope external glycoprotein (gp120) binding site is located in the D1 and D2 domains of CD4. Four charged residues (Lys-29, Lys-35, Lys-46, and Arg-59) and one hydrophobic phenylalanine at position 43 were essential for gp120 binding. These five amino acids are predicted to form a hydrophobic pocket by folding of the four charged amino acids around the hydrophobic phenylalanine residue, a structure that may be involved in direct contact with gp120. The MHC II and gp120 binding sites on CD4 are distinct but overlapping.

The HIV envelope precursor, gp160, is cleaved to yield gp120 and gp41 as mature envelope proteins. The HIV-1 gp120 envelope protein has a complex secondary structure stabilised by disulphide bonds between conserved cysteine residues. Extensive variability is apparent in five discrete gp120 domains termed V1-V5. More conserved amino acid sequences termed C1-C5 separate these hypervariable regions. Several of these domains interact with each other to form the complex secondary structure of gp120. Consequently, probing the CD4-binding site by amino acid deletions and substitutions in gp120 is often impeded by the extensive structural changes caused by the mutations. However, it has been established that the major regions of gp120 important for CD4 binding consist of residues 256 to 262 in the C2 domain, residues 368 to 389 in the C3 domain, and residues 421 to 457 in the C4 domain. Additional residues in the C5 domain have been reported to also contribute to the formation or stability of the CD4 binding

site. The C2 and C5 domains of gp120 are poorly exposed at the surface of the molecule (reviewed in Bour *et al.,* 1995) . It is thus likely that residues within these domains contribute to the structure of the CD4 binding site but are not involved in direct contact with CD4. In contrast, the C4 domain and most of the C3 domain protrude from gp120 and may be of particular importance for interactions with CD4.

HIV entry has been shown to be efficient at a neutral pH. In a model for virion entry into lymphoid cells, it is thought that conformational changes are induced in both gp120 and the transmembrane glycoprotein, gp41, after virions attach to cell surface CD4. The interaction of CD4 with the envelope glycoprotein promotes dissociation of the gp120 subunit from the gp41 subunit resulting in exposure of the hydrophobic fusion peptide at the N-terminus of gp41. After exposure by dissociation of gp120, the fusion peptide inserts into the lipid bilayer of the cell plasma membrane and thereby initiates fusion of the viral and cell membranes. Cell surface proteins other than CD4 may also induce conformational changes in the envelope glycoprotein.

Conformational changes in cell surface CD4 have also been reported following attachment of HIV (reviewed in Levy, 1993; Bour *et al.,* 1995). CD4 conformational change may be involved in T cell activation events important for virus transcription. A number of antibodies specific for domains of CD4 that do not participate in HIV binding have been shown to inhibit HIV replication in CD4 T cells. Antibodies against CDR3 loop of extracellular domain 1 have been shown to

inhibit HIV infection of CD4 cells without blocking either HIV binding or HIV entry into CD4 cells. The HIV neutralising activity of these antibodies was shown to be a consequence of their ability to inhibit virus transcription. Thus, certain epitopes within the CDR3 loop of CD4 appear to be important in the delivery of HIV-induced T-cell activation signals essential for HIV transcription. The identification of these novel HIV-neutralising antibodies has been central in establishing that CD4 acts as an essential signalling molecule in HIV infection.

3.2 Alternative receptors.

The T cell surface antigen CD4 is the principal binding site for the HIV virus, but alone is insufficient to allow entry of the virus into the cell (reviewed in Levy, 1993). This was first demonstrated when it was shown that mouse cells expressing human CD4 were not permissive for viral entry. Several alternative cell surface receptors have been implicated in virion attachment and entry into certain neuronal and colorectal tumour cell lines as well as several other cell types, all lacking detectable CD4. HIV-1 gp120 binds to galactosyl ceramide which is found on glial cell lines as well as colon cell lines. Complement receptors, such as Fc (fragment, crystalline) receptors offer another potential mechanism for viral attachment and internalisation. It is most likely that these adhesion molecules facilitate cell-to-cell contact during infection rather than functioning as true receptors which, by definition, must interact with a virion surface component.

Chemokine receptors as co-receptors for HIV entry

Immune cell trafficking comprises circulation, homing, adhesion, and recirculation of discrete populations of leukocytes between the blood vessels, lymph and lymphoid organ, and tissues. The molecular regulation of trafficking is complex, involving the interactions not only of cellular adhesion molecules such as selectins and integrins, but of an entire superfamily of chemoattractant cytokines (chemokines) and their receptors.

Chemokines can be classified into four families (C, CC, CXC, CXXXC) according to the number and location of cysteines and disulfide linkages (reviewed in Premack *et al.,* 1996). The most abundant are CC and CXC families which include macrophage inflammatory proteins (MIP-1α and MIP-1β) ,RANTES (CC) and stromal cell-derived factor-1 (SDF-1) and IL-8 (CXC). Structural distinctions of the different branches of the superfamily have been shown to parallel general distinctions in the biological activities of chemokines. For example, most CXC chemokines generally are chemoattractants for neutrophils but not monocytes, whereas CC chemokines generally attract monocytes, lymphocytes, basophils and eosinophils but not neutrophils. The C chemokine appears to be lymphocyte specific. PCR and blotting analyses suggest that most of the shared receptors are broadly distributed among leukocyte classes, but clear definition of tissue distribution of the receptors awaits wider availability of specific monoclonal antibodies.

Recently the first HIV co-receptors have been identified as members of the chemokine receptor family (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996). They are all seven transmembrane receptors from the large family of G protein coupled receptors, and include, CCR5, fusin, CCR3, and CCR2b. These co-receptors are responsible for bringing the surface membranes of the host cell and the virus together to allow fusion of the membranes and viral entry.

CCR5 is the chemokine which binds RANTES, MIP-1α and MIP-1β and is thought to be a co-receptor for macrophage tropic strains of HIV-1 (Deng *et al.,* 1996; Dragic *et al.,* 1996). A complex of gp120 of macrophage-tropic primary HIV-1 and soluble CD4 interacts specifically with CCR5 and inhibits the binding of the natural ligands. The apparent affinity of the interaction between gp120 and CCR5 was dramatically lower in the absence of soluble CD4. These results suggest that HIV-1 attachment to CD4 creates a high-affinity binding site for CCR5 leading to membrane fusion and virus entry (Trkola *et al.,* 1996; Wu *et al.,* 1996). The initial description of the CCR5 gene suggested that its expression is limited to granulocyte precursors, and absent in PBMCs. However, CCR5 transcripts were recently detected in both the monocyte/macrophage and macrophage depleted cell fractions of PBMCs (Deng *et al.,* 1996).

The fusin molecule is the co-receptor for T cell tropic strains of HIV. Fusin is a heterodimeric G protein coupled receptor and is related to the chemokine receptors (Berson *et al.,* 1996; Feng *et al.,* 1996). The sequence of fusin shows

greatest similarity with the CXC chemokine IL-8 receptor. Due to this homology, the term CXCR4 has been proposed for fusin, in keeping with current chemokine receptor terminology. The ligand for CXCR4 is SDF-1, which can inhibit HIV infection and the formation of syncytia by blocking the co-receptor (Oberlin *et al.,* 1996).

CCR3 is the receptor for the recently cloned chemokine eotaxin. Expression of the receptor CCR3 is more restricted than CCR5, being present on eosinophils, monocytes and T cells. Eosinophils also express CD4 and are permissive to HIV entry. Eotaxin was shown to inhibit CCR3 mediated HIV entry (Choe *et al.,* 1996; Doranz *et al.,* 1996).

Classification according to T cell tropic syncytium inducing strains of HIV-1 using CXCR4 and non-syncytium inducing macrophage tropic strains using CCR5 now appears to be over simplified (reviewed in Premack *et al.,* 1996). Macrophage-tropic strains may also use CCR3, while dual-tropic strains use CCR5, CCR3, CCR2b and CXCR4. Tropism of the virus may change with disease progression which may be to avoid suppressive activity of chemokines and to broaden the number of cells the virus can infect.

Subsequent to the discovery of CCR5 as a HIV co-receptor, Samson *et al* (1996) described a polymorphism in the gene for CCR5. The mutation is a 32 base pair deletion from the coding region and results in a frame shift. The gene product is a non-functional receptor that does not support membrane fusion or infection by HIV. The mutant allele of CCR5 is expressed at a high frequency in Caucasian

populations but is absent in black populations from Western and Central Africa and Japanese populations. In a cohort of HIV-1 infected Caucasian subjects, no individual homozygous for the mutant was found, and the frequency of heterozygotes was 35% lower than in the general population. White blood cells from an individual homozygous for the null allele were found to be highly resistant to infection by macrophage-tropic HIV-1 viruses, confirming that CCR5 is the major co-receptor for primary HIV-1 strains.

It has been known for some time that certain CD4-negative cell lines may be infected with HIV if large concentrations of the virus are used. In view of the interaction between certain chemokine receptors and gp120 it is possible that a high-titre virus stock is enough to allow the selection of variants that are better adapted to viral entry and replication in the absence of CD4 using a second receptor.

3.3 The immune response to HIV-1 infection

Primary HIV-1 infection is characterised by extremely high levels of viral replication. After the initial burst of viraemia viral titres decline dramatically, often as much as 10-100 fold. Immunological control of viral infection has been ascribed to multiple components of the immune response. Evidence exists to suggest that protection from infection or clearance of viraemia, or both, is mediated by natural killer cells that are non-specific for virus, virus specific antibodies, and cytotoxic T lymphocytes.

(i)Humoral immune response to HIV infection

The host defences against free virus particles involve neutralisation of infectivity, and occur either by antibody blocking HIV attachment or entry into a cell or by blocking uncoating of the virus (reviewed in Levy, 1993). Antibodies are also effective in mediating the destruction of virus-infected cells. This can occur by antibody mediated activation of the complement system, however this requires a high density of viral antigens at the cell membrane to prove effective. In contrast, antibody dependent cell mediated cytotoxicity (ADCC) (see below) mediated by natural killer (NK) cells can recognise as few as 10^3 IgG molecules in order to bind and kill the infected cell. Virus infected cells can also be destroyed via antibody-dependent cell-mediated cytotoxicity by macrophages and neutrophils.

The HIV envelope is the major target for humoral antibody responses. The viral proteins believed to be primarily involved in antibody neutralisation have been localised to the envelope gp120 and the external portion of gp41. Antibodies to the p17 core protein have also been reported to show neutralising activity. The presence of anti-HIV neutralising antibodies in infected individuals has been reported by several investigations and in general infected individuals have consistently demonstrated strong neutralising activity against their homologous strain. In general HIV-1 sera can neutralise HIV-1 but not HIV-2 strains. In contrast, sera from HIV-2 infected individuals have been reported to cross react with and neutralise some HIV-1 strains.

Most studies indicate that at least six regions of the viral envelope could be involved in HIV neutralisation (reviewed in Levy, 1993). A conserved domain within gp41 has been suggested and studies with hypervariable deletion mutants have also shown that domains in V1 or V2 and in V4 and V5 of gp120 could induce neutralising antibodies. An important neutralising domain of gp120, the principal neutralising domain (PND) is found in the central portion of the third variable region (V3 loop). Although V3 is a variable region, the PND is a linear epitope conserved among many strains or differing only slightly in amino acid structure. The V3-loop directed neutralisation does not involve the CD4-binding domain. The V3 loop can have both neutralising and non-neutralising epitopes, since sera with high-titre antibody to V3 peptides do not always neutralise the homologous HIV-1 strain. Moreover, work with escape mutants has indicated that regions within or outside the V3 loop can be involved in efficient antibody neutralisation. These sites outside of V3 can apparently alter the conformation of the V3 loop, permitting virion escape from neutralisation. Thus the V3 region can be both a linear and a conformational determinant for antibody recognition. Another major neutralising region on gp120 is in the CD4-binding domain, antibodies that cross-neutralise a large number of different strains, including those with different V3 regions, are directed against this region. The determinant is generally conformation dependent.

The clinical relevance of these antibodies remains uncertain. AIDS patients can have substantial titres of neutralising antibodies yet the virus would appear to

replicate unchecked. The virus may change under immunologic responses to escape neutralisation. However in the early stages of infection, antibodies can provide a major barrier to virus spread between cells and tissues and are important in restricting free virus in the plasma.

(ii) ADCC

Antibodies (IgG1 isotypes) to both the gp120 and gp41 envelope proteins induce ADCC. In this process, the antibody-antigen-coated cells are recognised by effector NK cells or monocytes Fc receptors and killed by a cytotoxic mechanism, most probably by perforins and secreted lysosomal enzymes. Only certain epitopes on HIV proteins induce this response, since not all anti-*env* antibodies produce this activity and they can be distinguished from neutralising antibodies. One detrimental effect of ADCC in HIV infection could be the release by cell destruction of large quantities of infectious particles with subsequent spread in the host.

(iii) Cell mediated immune response to HIV

(a) CD4 T lymphocytes

Antigen-specific T helper cells play an important role in retroviral infections (Levy, 1993). Indeed, they provide help for B-cell activation and antibody production and for clonal expansion of cytolytic lymphocytes. Upon antigen-derived activation, these cells release the appropriate lymphokines that induce clonal expansion of specific B cells and cytotoxic T lymphocytes (CTL). Activation of T

cells depends on recognition of antigenic peptides displayed by specialized antigen presenting cells (APCs), monocytes/macrophages, DCs and B cells. T helper cells are also involved in the recruitment and activation of macrophages at the sites of virus infection.

CD4 T cells can be separated into Th1 and Th2 subsets (Clerici *et al.,* 1994; Clerici *et al.,* 1997). Th1 cells secrete IL-2 and interferon-gamma (IFN-γ): Th2 cells produce IL-4, IL-6 and IL-10. Since the Th1 cells produce IL-2 and other cytokines that help in enhanced CD8 cell activity, this subset could also be important in the cellular immunological control of HIV infection and prevention of AIDS. A Th2 response results in B-cell activation and the secretion of high levels of IL-10 by the Th2 cells can lead to suppression of Th1 function. It has been demonstrated that Th1 responses are found primarily in healthy asymptomatic individuals and in high risk individuals who do not show evidence of HIV infection. Some investigators suggest that this type of response that helps induce cell-mediated immunity could be protective for an individual.

(b) CD8 Cytotoxic T Cells

Another cell type that commonly reacts with virus-infected cells is the CD8 cytotoxic T lymphocytes (CTL) (reviewed in Johnson *et al.,* 1994). Classically, this response is major histocompatibility complex (MHC) dependent and requires cell-to-cell contact. CD8 lymphocytes are an important feature of the immune response against HIV, eliminating virus infected cells. Resting CD8 lymphocytes are induced

to full effector function when presented with a variety of viral antigens, particularly to epitopes within the gag, reverse transcriptase, nef, and env proteins. CD8 cytotoxic T lymphocytes typically recognise short HIV peptide fragments expressed on the surface of infected cells in the context of a major histocompatibility class I (MHC-I) molecule and the target cell is killed. The target cell can be destroyed by inserting perforin pores in the cell membrane allowing the entry of enzymes called granzymes which leads to apoptosis or, if the target cell expresses the Fas molecule on its surface, the CTL may employ a Fas ligand to trigger apoptosis. On recognition of antigenic cells CD8 T cells secrete τ-interferon which induces resistance to virus in neighbouring cells and stimulates the activity of phagocytic cells.

(C) Anti-HIV suppressing activity of CD8 cells

CD8 T cells can also control HIV infection without killing the infected cells. This non cytotoxic cellular immune response involves suppression by CD8 cells of HIV replication (Levy *et al.,* 1996). This antiviral activity is not MHC restricted and is mediated at least in part by soluble factors. Infected individuals who were asymptomatic and whose cultured PBMCs did not yield HIV were studied and when CD8 cells were removed high levels of virus were released from the CD4 cells remaining in culture. Further observations indicated that the CD8 cells could suppress virus production without affecting activation markers on CD4 cells or killing the virus-infected cells. A soluble factor produced by the CD8 cells is

involved in this CD8 antiviral response. The presence of the factor can be shown by adding supernatants from CD8 cultures directly to infected CD4 cells. CD8 cells inhibit HIV replication before RNA transcription.

(d) Natural killer cells

NK cells mediate two forms of cytotoxicity: (i) lysis of virus-infected cells in a non-MHC restricted manner and without prior sensitization. (ii) Through CD16, NK cells can lyse IgG antibody-coated target cells; this mechanism is known as antibody dependent cellular cytotoxicity (Levy, 1993). NK cells may provide the first line of defence against viruses before activation of specific humoral and cellular immune mechanisms.

(e) Dendritic cells

Dendritic cells (DCs) are antigen presenting cells found in all tissues and organs of the body. Antigenically naive T cells are not widely distributed through the tissues, and are mainly found in the blood and lymphoid organs. DC are the principal antigen presenting cells (APC) involved in primary immune responses: their major function is to obtain Ag in tissues, migrate to lymphoid organs, and activate T cells. DCs are required for the stimulation of naive CD4 T cells, the development of potentially protective cytotoxic T lymphocytes, and furthermore, are more potent stimulators of secondary immune responses than monocytes (Caux *et al.,* 1995). Highly purified DCs can also stimulate a strong antibody response

against both the envelope and core proteins. In contrast, addition of adherent macrophages did not induce an HIV-specific antibody response (Roberts *et al.*, 1994).

3.4 Immunological abnormalities in AIDS

Infection with HIV-1 is persistent and associated with the development of profound immunosuppression. The principal immunological defect observed upon disease progression is the loss of CD4 T helper cells that play a role in all pathways of the adaptive immune response to pathogens. Uninfected individuals show a mean level of 1100 CD4 T cells/μl whole blood, while in AIDS CD4 T cell counts can as low as less than 1/μl. Theories about the causes of the observed loss of CD4 cells range from direct destruction or dysfunction by cytopathic infection with HIV-1 to apoptosis resulting from defects in antigen presentation (reviewed in Levy, 1993). Direct killing could be due to a cytolytic effect of the virus or to immune attack on virus infected cells. HIV can cause direct cytopathic effects in activated CD4 T cells in culture, either in single cells or by syncytium induction. Syncytia are giant multi-nucleated cells and may include as many as 500 CD4 expressing cells. These syncytia produce large quantities of virus for a short period of time and then die. By incorporating non-infected cells into syncytia, a single gp160 expressing cell can eliminate many uninfected CD4 cells. HIV-expressing cells will also be killed by HIV-specific cytotoxic T cell responses, which are the normal mechanism for eliminating virus infected cells. Antibody-dependent, complement

mediated cytotoxicity and other humoral immune effects may also help to remove HIV infected cells (reviewed in Weiss, 1993).

It has recently been demonstrated that the number of CD4 lymphocytes destroyed and replenished each day in HIV infection is of the order of 10^9, which was close to estimates of the total number of HIV-1 RNA expressing lymphocytes in the body (Ho *et al.*, 1995; Wei *et al.*, 1995). It may be that CD4 lymphocyte loss is primarily a consequence of the destruction of these cells induced by HIV while the gradual decline in CD4 and CD8 lymphocytes upon disease progression represents a failure of the host to adequately replace cells lost by infection (Ho *et al.*, 1995; Wei *et al.*, 1995).

Amongst indirect mechanisms, it has been demonstrated that *in vitro* HIV infection of lymphocyte cultures leads to a higher degree of apoptosis than normal (Finkel *et al.*, 1995; Gougeon *et al.*, 1996). If this also occurs *in vivo* it could account for T helper cell depletion. However it has been reported that the major cell type in the peripheral blood of HIV seropositive individuals undergoing apoptosis are uninfected CD8 T lymphocytes (Carbonari *et al.*, 1995). The depletion of CD4 T cells in AIDS patients shows similarities to the T-cell depletion that occurs following superantigen activation. Superantigens can bind simultaneously to a region of the T cell receptor and to a MHC class II molecule on an antigen-presenting cell. Uninfected cells which bind soluble gp120, may provide the necessary activation signal to initiate programmed cell death (Frost *et al.*, 1996).

The finding that the blood and lymph of AIDS patients contains large quantities of soluble gp120 is the basis for several other hypotheses to account for the depletion of uninfected CD4 T cells (reviewed in Levy, 1993). The noncovalent interaction of gp120 and gp41 is unstable, allowing large quantities of free gp120 to be shed into the surrounding fluid *in vitro*. If this phenomenon occurs *in vivo*, gp120 may bind to CD4 molecules on normal, uninfected CD4 T cells, thus blocking the interaction of CD4 with class II MHC molecules on antigen-presenting cells preventing the subsequent transduction of part of the activating signal. However very large quantities of soluble gp120 would be necessary to measurably block T cell function. Another possibility is that binding of soluble gp120 to CD4 membrane molecules may induce destruction of uninfected T cells by antibody-plus-complement lysis or by ADCC. Since numerous other cell types express CD4, these mechanisms might be expected to cause their destruction as well. However depletion of other CD4-bearing cells is much lower. The probable explanation of this difference lies in the density of CD4 molecules, which is considerably higher on CD4 T cells than macrophages or dendritic cells. Except for CD4 T cells, the density of CD4 molecules most likely is too low to induce ADCC or antibody-plus-complement lysis of other CD4-expressing cells. Binding of soluble gp120 to CD4 on an uninfected, activated T cell might result in receptor mediated endocytosis of gp120. The internalised gp120 might then be processed and presented together with class II MHC molecules on the cell membrane of the uninfected CD4 T cell. Although T cells that are CD4 and MHC class II restricted

generally function as helper cells, small numbers exhibit cytotoxic activity instead. These cytotoxic CD4 T cells as well as CD8 lymphocytes and natural killer cells could then selectively deplete these uninfected CD4 T cells.

Depletion of T cells normally induces T cell maturation within the thymus to restore the peripheral T cell numbers. Some researchers have suggested that soluble gp120 in AIDS patients binds to CD4 on thymocytes, thus interfering with the positive selection of class II MHC-restricted cells that occurs during T-cell maturation. T cell precursors in the thymus or peripheral pools may be infected with HIV and therefore fail to proliferate and replenish the mature T helper cell population (Schulzeosthoff *et al.,* 1993). It has also been suggested that defective antigen presentation by monocytes or dendritic cells would inhibit T cell proliferation (Knight, 1994).

It has been postulated that the T-cell homeostasis mechanism may fail preceding AIDS. Given the high rate of T-cell turnover in HIV infection, blind T cell homeostasis, where a constant number of lymphocytes is maintained without regard to CD4 or CD8 phenotype, if it exists would have important implications for HIV pathogenesis. This would account for the gradual shift in HIV infection from predominantly circulating CD4 lymphocytes to CD8 lymphocytes. The failure of T-cell homeostasis preceding AIDS would account for the depressed levels of total T cells (Margolick *et al.,* 1997).

The decline in CD4 lymphocytes and the initial increase in total CD8 counts in most HIV infected individuals is well documented (Pantaleo *et al.,* 1993).

However recent research has shown that CD8 naive cells are depleted during the asymptomatic stage of HIV infection and that this loss can parallel that seen in CD4 lymphocytes (Rabin *et al.*, 1995; Roederer *et al.*, 1995). CD4 naive lymphocytes are also lost preferentially as total CD4 counts fall. In individuals with CD4 counts less than 200 per μl, memory T cells were reported to account for 80% of T cells whereas they form only 15% of the CD4 population in uninfected individuals. Naive cells are required for all new T cell mediated immune responses and are memory precursors. Their loss, which precedes the eventual loss of memory cells, may contribute substantially to the eventual loss of the total CD4 and CD8 population. Loss of naive cells has important consequences for the development of immune responses in HIV infected individuals. As naive subsets disappear, there is a progressive inability to mount responses to novel antigens, which may well result in high susceptibility to opportunistic infections. This loss would also compromise the hosts ability to deal with the constantly mutating virus; novel strains which are immunogenetically unique will encounter less resistance from the host immune response than early in the progression of disease.

Even with a vigorous virus-specific CD8 response there is still the persistence of HIV viraemia, and high levels of CTL activity to HIV peptides does not prevent the development of disease. It is possible that virus infected cells are not recognised and conceivably, suppression of virus expression by the non-cytotoxic CD8 cell antiviral factor could contribute to this lack of recognition by CTL. Some recent reports suggest that HIV can escape the CTL response and this mechanism could be involved in progression to disease (reviewed in Johnson and Walker, 1994). A decline in the number of circulating CD8 lymphocytes, a reduction in the cytotoxic response and loss of CD8 T cell function have been reported in patients with more developed disease status (Edwards, 1972). The cell loss and decline in cell function can be observed in healthy seropositive individuals and occurs over time as the individuals CD4 count decreases and the disease progresses. This loss of immune function may contribute to the rapid fall in lymphocytes observed as an individual approaches AIDS (CD4 $<200/\mu l$).

A major component of cellular immunity is the NK cell, which recognises and kills virus-infected cells in a non-MHC-directed manner. Impaired NK cell activity and the decline in NK cell numbers is one of the many immunological defects observed in patients with AIDS (Mitchell *et al.*, 1994; Brenner *et al.*, 1997).

A block in the capacity of DC from the peripheral blood to stimulate T-cell proliferation after exposure to HIV is a feature of cells from HIV-infected persons. DCs from asymptomatic HIV-seropositive individuals showed a reduced capacity to stimulate either naive or memory T-cell responses as assessed from stimulation

of allogenic mixed leukocyte reactions and recall responses in autologous T cells. Findings indicate that both naive and memory T cells and monocytes were functioning normally in these individuals and pinpointed the defects to signalling through the DCs (Macatonia *et al.,* 1990). The later appearance of T cell defects is compatible with the thesis that they may be secondary to loss in the function of DCs.

The decline of CD4 T helper cells in AIDS patients eventually affects the functioning of B cells in the humoral response (Lane *et al.,* 1983). As AIDS progresses, patients are increasingly unable, from lack of T helper cells to mount a humoral antibody response to new antigens. This may be important in relation to the antigenic drift of HIV. As the virus mutates in HIV infection, late stage individuals may not mount an adequate antibody response against the new HIV variant.

3.5 Evidence for *in vivo* and *in vitro* infection of different cell subsets with HIV-1.

It has until very recently been generally accepted that the cellular tropism of HIV is determined almost exclusively by the distribution of CD4, the cell surface receptor for HIV-1. CD4 is expressed on T helper cells, monocytes and peripheral blood dendritic cells (Freudenthal *et al.,* 1990) but not on mature cytotoxic T cells, natural killer cells or B lymphocytes.

(i) Infection of CD4 and CD8 lymphocytes with HIV-1.

HIV-1 infection of CD4 lymphocytes has been reported both *in vitro* and *in vivo* (Schnittman *et al.,* 1989). In fact CD4 lymphocytes are thought to be the major reservoir of HIV-1 infection within the peripheral blood of seropositive individuals. Early reports indicated that from 1 to 0.01% of CD4 T Cells contained viral DNA, (Psallidopoulos *et al.,* 1989; Schnittman *et al.,* 1989). However a more recent study using a sensitive PCR technique has suggested that at least 10% of CD4 lymphocytes within the peripheral blood harbour HIV-1 *in vivo* (Hsia *et al.,* 1991).

Mature CD8 lymphocytes in the peripheral blood do not express CD4 at their cell surface, however numerous studies have reported the infection of CD8 lymphocytes *in vitro* (Tsubota *et al.,* 1989; De Maria *et al.,* 1991; Mercure *et al.,* 1993). De Maria *et al* demonstrated that CD8-positive, CD4-negative lymphocyte lines derived from infected individuals express HIV proteins and generate reverse transcriptase activity (De Maria *et al.,* 1991; De Maria *et al.,* 1994). Infection was confirmed at a single cell level by immunoelectron microscopy and two colour immunohistochemistry. Seven days after establishing such cultures approximately 5% of lymphocytes co-expressed CD4 and CD8. The basic requirements for HIV infection of CD8 lymphocytes was the presence of CD4 cells. PCR of sorted cells revealed that the CD8 cells harboured HIV-1 proviral DNA and stimulation of these cells resulted in virus replication and infection of CD4 cells. This study indicated that CD8 lymphocytes of patients with late stage AIDS may be infected

with HIV-1 but the findings were not published. Mercure *et al* found that CD8 cells can be productively infected when co-cultured with HIV-1 infected CD4 positive cells. However direct infection of purified CD8 lymphocytes with isolated virus was not possible (Mercure *et al.,* 1993).

Infection of lymphoid CD4 and CD8 cells by SIV has been demonstrated by DNA PCR and by cell sorting and virus isolation by co-culture (Dean *et al.,* 1996). In this study as many as 19.4% of the positively selected CD8 cells also expressed CD4, indicating that SIV provirus may reside in dual positive cell populations and that this may provide a mechanism of entry for HIV into CD8-positive, CD4-negative cells *in vivo.* Semenzato and colleagues reported that CD8 lymphocytes in the lung of AIDS patients harbour HIV-1. PCR of lung CD8 lymphocytes retrieved by bronchiolar lavage showed that they harboured and expressed HIV-1 (Semenzato *et al.,* 1995). Although there was a lack of membrane CD4 reactivity it was demonstrated that the lung CD8 cells could express CD4 RNA.

Possible mechanisms for HIV infection of CD8 lymphocytes *in vitro* include: (a) Viral transmission through cell-to-cell contact. This has already been reported between monocytes or dendritic cell and CD4 lymphocytes and between CD4 lymphocytes (Valentin *et al.,* 1990; Cameron *et al.,* 1992; Levy, 1993; Cameron *et al.,* 1997). It has also been demonstrated that the minimum requirement for infection is the presence of CD4 cells. (b) CD8 T cell activation by PHA may up regulate CD4 expression at the cell surface allowing infection. An increase in

double positive lymphocytes upon PHA stimulation has been reported (Flamand *et al.,* 1997). (c) A CD4 independent mechanism using a chemokine receptor for virus entry.

(ii) Macrophages

Monocytes/macrophages which express the CD4 receptor for HIV, are often considered an important reservoir of this virus in infected patients. HIV does not replicate as actively as in CD4 T lymphocytes and HIV DNA rather than RNA can be detected (Levy, 1993).

In the spleen HIV is found mostly in CD4 lymphocytes and trapped on the surface of follicular DCs, while macrophages are not the major target population. The results obtained in the spleens of HIV patients are comparable to those obtained in the peripheral blood. Infection of macrophages was 20-100 times lower than in CD4 T lymphocytes, which contained practically all the viral load of the unseparated mononuclear cell populations (Mcilroy *et al.,* 1996).

Although peripheral blood monocytes express CD4 and the activation marker HLA-DR there is little evidence for their extensive infection with HIV (Collman *et al.,* 1990; Valentin *et al.,* 1991; Levy, 1993). Estimates of the frequency of infected monocytes ranged from zero to $100/10^6$ cells (Schnittman *et al.,* 1989; Innocenti *et al.,* 1992; Bagasra *et al.,* 1993; Hsia *et al.,* 1995). Such results have collectively led to the view that the ability to support HIV replication increases as monocytes differentiate into macrophages even though CD4 surface expression

decreases with such differentiation. Evidence that differentiation into macrophages is required for productive infection was obtained by comparing monocyte culture in suspension rather than adherance to plastic and by the increased susceptibility to HIV-1 that occurs on culture of monocytes in the presence of the maturation inducer granulocyte-macrophage colony stimulating factor (GM-CSF) (Valentin *et al.*, 1991).

Separate studies have suggested that monocytes are either susceptible to HIV in the absence of detectable CD4 on their surface or express CD4 on only a small proportion of cells which are then susceptible to infection. In one study, CD4 antibody completely inhibited HIV infection of alveolar macrophages indicating that CD4 although expressed at a low level appeared to be critical to HIV infection of alveolar macrophages (Lewin *et al.*, 1996). However, Sonza *et al* (Sonza *et al.*, 1995) concluded that the susceptibility of human monocytes to HIV-1 infection *in vitro* is not dependent on their level of CD4 expression. Observations suggest that the differing susceptibility of monocytes and monocyte-derived macrophages to infection with HIV is not simply proportional to the level of surface CD4 expression.

The reasons why monocytes are resistant to HIV-1 infection are unknown. CD4 on monocytes is reportedly identical to that on lymphocytes and thus gp120 binding properties should be the same (Stewart *et al.*, 1986). However, the CD4 molecule on monocytes has been has been found to have reduced binding affinity for gp120, when compared with binding of gp120 to CD4 on macrophages

(Finbloom *et al.,* 1991). Binding to an accessory receptor, such as CCR5, the expression of which increases with culture may contribute to viral attachment and entry, thereby facilitating infection of the cultured cells. There is currently no evidence available to support this theory.

(iii) Dendritic cells

Although dendritic cells and macrophages share a bone marrow origin, these cells were long assumed to differentiate via discrete pathways. DCs have now been shown to develop from myeloid lineage precursors, and recent evidence suggests that they may even differentiate from blood monocytes (Peters *et al.,* 1996). Purified fresh peripheral blood DCs lack the characteristic morphology, phenotype and immunostimulatory function of mature DCs. The purified cells have the appearance of medium sized lymphocytes and express substantial levels of CD4, but lack the T cell molecules CD3, CD8, and T cell receptor (TCR). When placed in culture, the cells mature: the cells enlarge and exhibit many cell processes, express much higher levels of MHC II and a panel of accessory proteins for T cell activation, and become potent stimulators of the mixed leukocyte reaction. Among the many changes during the maturation process is a fall in CD4 expression (O'Doherty *et al.,* 1993).

Peripheral blood dendritic cells can be infected *in vitro* with HIV-1. However, increased levels of infection were produced on culture with the maturation cytokines, GM-CSF and tumour necrosis factor-alpha (TNF-α), and more proviral

copies than cells were obtained in the cultures. In common with monocytes, DCs appear more susceptible to HIV infection as CD4 levels decline or are lost (reviewed in Knight, 1996).

DC purified from human peripheral blood were inoculated with various strains of HIV-1, and viral replication was demonstrated by detection of p24 Ag, and by Southern and Northern blot analyses for the presence of HIV DNA and RNA. Purified DC did not express detectable membrane CD4, although CD4 mRNA was detected by RT-PCR. The presence of anti-CD4 monoclonal antibodies failed to block infection of DC by any of the HIV strains tested, suggesting the existence of a CD4-independent alternative pathway of viral entry (Chehimi *et al.*, 1993).

Hsia *et al* (1995) were unable to detect HIV-1 DNA in 10^4 or 10^5 DCs. Cameron *et al* detected HIV DNA in $5x10^5$ dendritic cells but these were thought to be contaminated with demonstrable numbers of CD4 lymphocytes. However, contradicting these findings, DCs have been isolated from the blood of individuals in different clinical categories and HIV infection has been demonstrated (Patterson *et al.*, 1994). The frequency of cells harbouring HIV DNA in purified populations of cells isolated from the spleen were quantified by limiting-dilution PCR. Directly isolated DC in each case were 10-100 times less infected than CD4 lymphocytes. DC seem to be infected, but HIV-DNA positive CD4 lymphocytes accounted for the vast majority of infected mononuclear splenocytes (Mcilroy *et al.*, 1995).

Evidence suggests that DC become productively infected only as they mature *in vivo* (Weissman *et al.,* 1995). Within peripheral blood, three forms of DC at different stages of maturation were observed by electron microscopy. These cells ranged from those with short cytoplasmic projections, the majority of cells in the peripheral blood exhibited this morphology, to the occasional completely veiled DC. HIV was never detected in the more immature DCs but in the fully veiled cells, electron microscopy revealed viruses budding from the cell membrane or in clusters attached to the cell surface.

The rate and efficiency of key steps in the life cycle of HIV-1 was examined in three primary cell types, T cells, DCs, monocytes (Langhoff *et al.,* 1993). The results show that viral DNA synthesis proceeds much more rapidly and efficiently in primary DCs than in primary T cells or monocytes. In the subsequent phase of viral expression the appearance of spliced viral mRNA products also occurred more rapidly in cultures of DC. The increased efficiency of the early steps of HIV replication in primary blood derived T helper DC than in other blood derived mononuclear cells raises the possibility that these cells play a central role in HIV-1 infection and pathogenesis. The high level of virus produced by populations of DCs is remarkable as these cells are terminally differentiated and do not proliferate in culture. However other studies would suggest that any DCs may have latent or non-productive infection with virus, since a much higher proportion of DC contained viral DNA than expressed viral RNA.

(iv) Natural killer cells

Among other defects, patients with AIDS display abnormalities in NK cell activity and NK cells decrease in HIV infection through a selective depletion of the CD16-positive, CD8-positive, CD3-negative subset (Vuillier *et al.,* 1989). Recent results indicate that purified populations of NK cells, which express neither surface CD4 nor CD4 mRNA, were susceptible to infection with various isolates of HIV-1 (Chehimi *et al.,* 1991; Scottalgara *et al.,* 1993). Viral replication was detected by p24 antigen intracellularly and in culture supernatants, by the presence of HIV DNA within infected cells. Infection of NK cells was not blocked by anti-CD4 suggesting a CD4 independent mechanism of entry was involved. Infection was only detected in the subset of NK cells expressing CD8.

It is possible that HIV infection of NK cells could be involved in the decline in their number and function observed in AIDS patients. In order to investigate this NK cells were compared from infected and uninfected cultures. NK cells from HIV-infected and uninfected cultures were similar in their ability to lyse three different target cells. However, considerable numbers of NK cells in the infected cultures died, suggesting that the loss of NK cells in HIV infection is as a result of virus replication but that reduced NK cell function involves another mechanism. The possibility that NK cells serve as a potential reservoir for HIV-1 *in vivo* must be considered (Chehimi *et al.,* 1991).

NK cells were exposed to different strains of HIV-1. IIIb and SF2 strains were able to infect NK cells but less infection was observed when using a brain

isolate. Two viruses isolated from lymphocytes of children with AIDS were able to infect NK cells but 3 isolates from monocytes of children with AIDS were unable to infect purified NK cells. Since the five isolates have not been fully characterised it remains to be determined whether NK cells are differentially permissive to lymphocyte-tropic or monocyte-tropic strains of HIV-1 (Chehimi *et al.*, 1991).

3.6 Viral dynamics of HIV infection

In general retroviruses can productively infect only actively dividing cells. In contrast, lentiviruses, including HIV-1 can establish a productive infection in certain non-dividing cells (Watkins *et al.*, 1990). It has been demonstrated that HIV-1 can infect and establish a complete, stable form of viral DNA in primary CD4 lymphocytes *in vitro* but is blocked from transcription in the absence of cell activation. Thus quiescent T cells may be a major and inducible reservoir in infected individuals (Spina *et al.*, 1995).

It was show by virus quantitation and mutation fixation rates that the composite lifespan of plasma virus and of virus-producing cells is remarkably short. This holds true for patients with CD4 counts as low as 18 cell per μl and as high as 355 cells per μl. Investigations estimate that the rate of CD4 lymphocyte turnover is on average, $2x10^9$ cells per day, or about 5% of the total CD4 lymphocyte population, depending on clinical stage, which is close to the number of lymphocytes expressing HIV mRNA in the body (Ho *et al.*, 1995; Wei *et al.*, 1995). The data suggest that HIV-1 viraemia is sustained by a dynamic process

involving continous rounds of *de novo* virus infection and replication and cell turnover and not an 'innocent bystander' mechanism of cell killing whereby uninfected or latently infected cells are indirectly targeted for destruction by adsorption of viral proteins or by autoimmune reactivities .

3.7 How does HIV cause AIDS?

How does HIV cause AIDS? There are no definite answers but there are numerous theories discussing how HIV may lead to AIDS and these are summarised below (reviewed in Weiss, 1993).

It has been hypothesised that the chronic activation of the immune system occurring throughout HIV infection is the mechanism responsible for the cell deletion process. By the onset of AIDS antibody responses are low and APC had little effect. This might reflect the significant T-cell depletion and dysfunction that occurs with AIDS and thus a lack of T cell help. The immunological abnormalities observed may be, in fact secondary to CD4 T helper cell loss, as these cells are directly involved in many areas of the host immune response to viruses.

The powerful immune response enabling many patients to remain healthy for years may finally be undermined by continuous mutation of the virus (Vazeux *et al.*, 1992). New viral variants may emerge that are able to evade the protective forces some what. These features enable the virus to evolve in response to the threats it encounters during the course of an individual infection. Mutants able to evade immune attack to some degree appear and predominate until the immune

system gathers the strength to quell them, but meanwhile new escape mutants begin to multiply. Generation of mutants thus stimulate a continuous reduction in the efficiency of the immune system. At some point, the diversity becomes too extensive for the immune system to handle, and HIV escapes control. More cytopathic variants of HIV may possibly arise as the immune system becomes weaker increasing the rate of cell loss in AIDS.

The preferential loss of naive cells has important consequences for the development of immune responses in HIV infected individuals (Schulzeosthoff *et al.,* 1993; Rabin *et al.,* 1995; Roederer *et al.,* 1995). As naive subsets disappear, the host becomes less able to control opportunistic infections and new virus variants. These naive cells are also precursors for the memory population and thus their loss will inevitably lead to a reduction in the overall number of circulating T lymphocytes. Given the high rate of T-cell turnover in HIV infection, blind T cell homeostasis would account for a shift from CD4 to CD8 lymphocytes and a failure of this mechanism would account for the depressed level of total T cells (Margolick *et al.,* 1997).

Specific cellular immune responses are driven by various cytokines. Immunoregulatory cytokines are produced by T cells, monocytes/macrophages, natural killer cells and B cells. A type 1 response is a strong cellular immune response with normal or increased levels of IL-2, IL-12 and interferon-gamma (IFN-γ); and a type 2 response as a reduced or undetectable cellular response accompanied by an increase in one or more B-cell activities and an increase in IL-4,

IL-5, IL-6, IL-10 and/or IL-13. Recent studies indicate that type 2 cytokines are increased in HIV infected individuals in the progression towards AIDS (Clerici *et al.,* 1994; Clerici *et al.,* 1997). A dominant type 1 cytokine profile would be more protective against disease progression than a dominant type 2 cytokine profile. It has been shown that HIV-specific cellular immune responses, can be detected in cohorts of individuals multiply exposed to HIV, many of whom do not appear to be infected. Thus a type 1 to a more dominant type 2 response in HIV infection could be instrumental in the failure of the immune response to cope with HIV replication in the later stages of disease. A type 1 to type 2 switch has been noted with progression to AIDS in some individuals.

Studies investigating the infection of DCs with HIV have suggested that DC infection is in fact important in the development of AIDS (Knight, 1996). Studies have revealed that DCs are functionally impaired during asymptomatic HIV infection before the development of T-cell abnormalities. DCs from asymptomatic HIV-seropositive individuals showed a reduced capacity to stimulate either naive or memory T-cell responses as assessed from stimulation of allogenic mixed leukocyte reactions and recall responses in autologous T cells. Loss or dysfunction of DCs impairing the recruitment of T helper cells into the memory pool would contribute to the progressive decline in CD4 lymphocytes. Loss of DC function may also be important in other areas of immune abnormalities in AIDS. Examples of the important roles of DCs which may be lost or impaired are the stimulation of a

cytotoxic T cell response and their involvement in the development of an antibody response (Roberts *et al.*, 1994).

Interacting DC and helper CD4 lymphocytes form a micro environment which is permissive for HIV-1 replication (Cameron *et al.*, 1992; Cameron *et al.*, 1997). The virus need only be pulsed initially onto the DCs, which then transfer HIV-1 to the lymphocytes that are responding to the presented antigens. Pulsing T cells with HIV-1 results in much less of a subsequent infection than does pulsing the DCs. Direct examination of the interacting DCs and T cells reveals extensive production of p24 by many of the lymphocytes including syncytia. The majority of the responding T cells die during the co-culture. Apoptosis accounts for much of this death. Thus the micro-environment that is generated between antigen presenting DCs and T cells reveals the cytopathic potential of HIV-1, because there is such extensive and rapid death by apoptosis of the antigen-reactive T cells. These observations could be important in the lymph node of infected individuals where DCs come into close contact with many T cells.

DCs from the thymus may be infected with HIV as well as thymic T cells (Knight, 1996). Changes in the function of DCs within the thymus could alter the selection of T cells and be a factor in the loss of naive T cells that occurs in AIDS. Failure of DC from infected individuals to stimulate proliferation of naive T cells could underlie the loss of memory cells that also occurs. Within the spleen and lymph nodes only low levels of infection of DCs have been described.

Finally is it possible that immune dysfunction and the cell depletion observed in AIDS is directly due to the cytopathic effects of HIV infection. Early reports claimed the HIV infection was silent during the asymptomatic stage. However recent reports would suggest that the asymptomatic stage is in fact far from silent with the immune system battling to replace cells lost to HIV replication. It has been demonstrated that the number of CD4 lymphocytes destroyed and replenished each day is of the order of 10^9 which was close to estimates of the total number of HIV-1 RNA expressing lymphocytes in the body (Ho *et al.,* 1995; Wei *et al.,* 1995). It may be that CD4 lymphocyte loss is a consequence of the destruction of these cells induced by HIV.

Direct cell loss due to HIV replication is further supported by investigations of lymph node infection (Embretson *et al.,* 1993). Using *in situ* PCR it was possible to visualise the distribution of HIV RNA and DNA in the lymph nodes and to show that cells in the lymph nodes are infected at ten times the frequency of cells in the peripheral blood. Because the lymphoid organs contain the majority of T cells, while the peripheral blood contains only a small fraction (about 2% of all T cells). Emberetson *et al* (1993) and showed that HIV is not only abundant, but also active in the lymphoid tissue during the asymptomatic phase, even though the virus in peripheral blood was inactive at this time. This offers an explanation for the loss of CD4 cells during the asymptomatic phase when the number of infected cells in the peripheral blood is relatively low. In the later stages of disease the architecture of the lymph nodes collapses, and these organs loose their ability to trap the virus.

The spillover from the lymphoid organs could partially explain the increase in viral burden as the disease progresses.

3.8 Cell Separation

Results *in vivo* suggest that a much wider range of cells are infectable with HIV than have been demonstrated *in vivo*. Previous studies have also demonstrated that cells which do not express CD4 can be infected. Therefore the host cell range of HIV-1 within the peripheral blood of seropositive individuals may be broader than those cell types identified, that is CD4 T lymphocytes, monocytes, and dendritic cells. The aim of this investigation was to determine the *in vivo* distribution of HIV-1 proviral sequences in different cell subsets of PBMCs isolated from seropositive individuals at different stages of disease progression. Cell subsets were isolated using three different isolation methods, and HIV-1 DNA was quantified in each fraction using primers complementary to the *gag* and V3 regions of HIV-1.

The underlying problem with any investigations where individual cell types are isolated from PBMCs is that cells are not a homogenous population and different purification procedures may result in variations in the purities of the cells isolated. The cell isolation procedures alone may cause cell activation or maturation altering the cells from their *in vivo* state. In the main PBMCs are isolated by layering whole blood onto ficoll hypaque, centrifugation and harvesting the cells from the interface. Unfortunately this first step activates the monocytes present.

Different groups have used various techniques to isolate DCs and these are summarised below. Freudenthal *et al* (1990) separated T lymphocytes by rosetting with neuraminidase-treated sheep erythrocytes followed by ficoll-hypaque sedimentation. T cells were recovered from the pellet by lysing the erythrocytes and washing twice in RPMI. The T-cell depleted (ER-ve) fraction was washed twice in RPMI and cultured for 36 hours. By reculturing the cells twice for 30-40 minutes at 37°C on fresh dishes monocytes were depleted by attachment to plastic. Non-adherent cells were then 'panned' once or twice on plastic dishes coated with immunoglobulin to remove residual Fc fragment receptor (FcR)-bearing monocytes. The monocyte and T-cell-depleted fraction was layered onto 2.5 ml columns of hypertonic 14.5% metrizamide in 15 ml conical tubes and sedimented at 650 x g for 10 minutes at room temperature. The dendritic-cell-enriched interface was separated from the B and natural killer (NK) enriched pellet.

Patterson *et al* (1994) incubated PBMCs overnight to provide plastic adherent and non-adherent cells. The non-adherent cells were centrifuged over 13.5% weight/volume metrizamide to separate the lymphocytes and low density cells (LDC). It was estimated that 10-40% of these LDCs were DCs. The LDCs were incubated with a cocktail of monoclonal antibodies directed against CD3, CD14, CD56, and CD19 (T cells, monocytes, natural killer cells, and B cells respectively). Labelled cells were removed by panning on anti-mouse immunoglobulin-coated flask. By flow cytometry it was estimated that the final DC purification was 95% pure.

Chehimi *et al* (1993) concluded that differences in results might be explained by the different methods used to purify DC. They found that the use of 14.5% metrizamide yielded preparations contaminated by monocytes.

Initially a procedure was developed which involved the selection of CD4 T cells, CD8 T cells, monocytes and B cells. Dendritic cells were then positively selected from the depleted fraction. The use of metrizamide and cell culture techniques were avoided. Lymphocytes were first isolated by rosetting with neuraminidase treated sheep red blood cells. The T cell fraction could then be separated into CD4 T cells and CD8 T cells with monoclonal antibodies. E-rosette negative (ER-ve) cells were incubated with a series of monoclonal antibodies, first with CD14 (monocytes), then CD21 (B cells), and finally with CD4 and MHC-II HLA-DR (Dendritic cells). Each monoclonal Ab was pre-bound to magnetic beads so each cell type could be fractioned using a magnet after each incubation.

DCs are difficult to purify partly because they are present in such small numbers, and because no DC-specific cell markers have been identified (Freudenthal *et al.,* 1990). Furthermore it has been reported that a population of DCs are lost in the E-rosette positive fraction either because they directly bind sheep red blood cells or because they cluster with T cells. Weissman *et al* (1995) isolated three populations of dendritic cells using various techniques. Only one type of these isolated DCs were infected with HIV. Thus discrepancies in findings of different groups studying dendritic cell infection could be due to different isolation procedures. Dendritic cell isolation proved to involve many disadvantages, most

prominent was the level of cell loss that resulted from the E-rosetting procedure. E-rosette positive cells were also found to contain natural killer cells which can express CD2 and therefore bind to the SRBCS. Therefore in later cell isolation procedures, the miniMACS cell separation method was used, the dendritic cell fraction was not isolated and the methods used concentrated on the isolation of other cell subsets.

Another disadvantage of the first technique was that the beads used could not be directly FACS analysed so cell purity could only be inferred. The second cell separation method involved the use of MiniMACS directly conjugated beads and separation columns. MACS Microbeads are extremely small, only 50 nm in diameter and can be directly analysed by FACS as they do not affect the light scattering of labelled cells. The purity of the sorted fractions can be determined directly after MACS separation by flow cytometry and labelled cells can be stained simultaneously with fluorochrome conjugated antibodies. The size and composition of these beads mean they are biodegradable and were therefore useful where cells were required for cell culture as they do not alter the cell physiological function. The MiniMACS system was already optimised and the manufacturer claimed that typically isolated cells were of 95-99% purity. Monocytes, CD4-positive cells, and natural killer cells were first isolated before anti-CD8 beads were used to purify CD8 T lymphocytes. However, because the E-rosetting method was not used (in order to limit cell loss during cell separation), T cells were not first isolated, therefore the CD4 T lymphocytes population was contaminated with DCs.

Finally CD4 and CD8 cells were isolated from four individuals using Dynabeads. This study was carried out in order to further verify the PCR results. In order to do this two positive and two negative samples were separated in an independent lab (St Marys Hospital, London) and were numbered anonymously. PCR reactions were then carried out blind.

RESULTS

3.9 Cell Separation and FACS Analysis

30 mls of whole blood were taken from 34 HIV seropositive individuals with CD4 counts ranging from less than 1 to 938 /μl of blood. Of the individuals studied 19 had CD4 counts less than 200 and 12 had CD4 counts of greater than 200. There was also an even distribution of individuals infected by heterosexual contact, homosexual contact and intravenous drug use. Other virological and immunological data for the individuals studied are given in tables 2.1 and 2.2. Cell subsets were isolated from PBMCs using 3 purification methods; (i) Using an E-rosetting technique, E-rosette positive cells were first incubated with anti-CD4 coated magnetic beads to isolate CD4 T cells. The deleted fraction was then treated with anti-CD8 in order to positively select CD8 T lymphocytes. Monocytes, B cells and DCs were sequentially purified from the E-negative fraction using magnetic beads coated with CD14, CD19 and CD21, and CD4 and HLA-DR. Blood samples from 17 individuals with CD4 counts ranging from <1 to 938/μl blood, were treated in

this manner (individuals coded P). None of these individuals were undergoing anti-retroviral therapy or had received treatment in the previous six months (Table 2.1). (ii) MiniMACS beads directly conjugated to anti-CD14, CD4, CD56 and CD8 were used to select monocytes, CD4 lymphocytes, NK cells and CD8 T lymphocytes respectively. This isolation method was carried out on 15 samples from individuals with mean CD4 counts ranging from 3 to 420/μl blood. 5 of these individuals were undergoing anti-retroviral therapy. (individuals coded S04-S26, Table 2.2) (iii) In a blind study with two HIV positive (Anon1 and 2) and two negative control samples Dynabeads conjugated with ani-CD4 and CD8 were used to purify CD4 and CD8 lymphocytes.

For each cell separation method negative control PBMCs were separated in parallel. Aliquots of isolated cells were then FACS analysed using a panel of monoclonal antibodies, in order to determine purity. For raw data see appendix.

The process of isolating subsets of mononuclear cells using indirect labelling of magnetic beads (Immunotech) prevented analysis of the selected cells by FACS techniques. However, the purity of cell fractions could be inferred by measurement of the frequencies of different cell types in the residual cells after removal of a particular fraction. Mean values for each cell fraction were calculated from FACS data of three negative controls run in parallel with positive samples. The E positive cells (T cell fraction) contained a mean frequency (3 samples) of 82.8% CD3 cells (Table 3.1). Contaminating cells included B lymphocytes (0.9% CD19 cells) and natural killer cells (15.5% CD16 cells). The presence of NK cells was

Table 3.1 : Purity of cell fractions isolated using the E-rosette method and monoclonal antibody coated magnetic beads by FACS analysis.

		% Cells				
Subset removed	**Monoclonal**	**CD3** [b]	**CD4** [b]	**CD8** [b]	**CD16** [b]	**CD19** [b]
(a) E rosette positive [c]						
E positive	None	82.8	47.0	40.1	15.5	0.9
CD4 T cells	CD4	54.4	1.7	54.5	14.5	ND[a]
CD8 T cells	CD8	12.0	4.4	4.2	12.7	ND
Subset removed	**Monoclonal**	**CD3** [b]	**CD4** [b]	**CD14** [b]	**CD21** [b]	**HLA-DR** [b]
(b) E rosette negatives [d]						
E negatives	None	1.0	64.7	59.5	8.6	ND
monocytes	CD11b/CD14	1.0	37.8	3.7	ND	ND
B cells	CD19/CD21	1.0	43.5	3.7	ND	53.2

a ND= Not Done

b CD3, CD4, CD8, CD19, CD16= Monoclonal antibodies preconjugated to fluorescent dyes used for FACS analysis.

c E rosette positive fraction consists of T cells. Mean frequencies of three separate purifications).

d The E rosette negative population are non-T cells (B cells, monocytes and dendritic cells); results from a single sample.

expected as they express CD2 which binds to SRBCs. Positive selection for CD4 cells was shown by a reduction of CD4-positive cells from a mean of 47.0% in the E-rosette (ER) positive population to 1.7% in the depleted cells. There was also a reduction in the number of CD3 positive cells from 82.8% to 54.4% and an enrichment of CD8 positive cells. CD4-depleted cells consisted of 54.5% CD8 cells, which were then positively selected on CD8-coated beads. Residual cells after CD8 depletion contained only 4.2% CD8 cells and 12.0% CD3 cells. There was a small decline in NK cells from 14.5% to 12.7% as these cells can express low levels of CD8. The remaining CD4-negative, CD8-negative, CD3-negative cells contained NK cells, dead cells and any remaining SRBCs. ER-ve cells (non-T cells) (table 3.1) contained less than 1% CD3-positive T cells. These cells were first incubated with anti-CD3 coated beads to remove these residual T cells. Monocytes were effectively removed by means of beads coated with CD11b and CD14 (59.5% to 3.7%). The remaining cells were depleted by use of CD19-coated and CD21-coated beads to remove residual B cells. Less than 1% of the resulting population expressed CD3 while, 43.5% expressed CD4, and 53.2% HLA-DR, a phenotype consistent with dendritic cells. Magnetic beads coated with anti-CD4 and anti-HLA-DR coated magnetic beads were then used to isolate the dendritic cell fraction. DCs express no cell surface markers (Freudenthal *et al.*, 1990) so it is difficult to determine their purity by FACS analysis. Therefore the purified DCs from negative control samples were resuspended in cell culture medium and incubated at 37°C. The cultured cells were analysed by light microscopy and after 5 days cells

with a veiled morphology were detected. This morphology is characteristic of a mature DC (Freudenthal *et al.,* 1990).

When this E-rosetting technique followed by positive selection of cells using monoclonal antibody coated magnetic beads was used, direct FACS analysis of the positively selected cells was not possible and therefore the level of cell purity had to be inferred from depleted cell fractions. The first method does not necessarily prevent analysis of subset purity and the selection steps could be carried out using miniMACS beads. However a further problem with the E-rosette technique was the substantial cell loss observed, especially when lysing the sheep red blood cells. Therefore the E-rosetting step was omitted in later procedures

CD14, CD4, CD56 and CD8 positive cells from 15 individuals were purified using directly coated MiniMACS beads and columns. FACS analysis was carried out using six HIV positive samples and five negative control samples for monocytes, CD4-positive cells and CD8-positive cells. Only three natural killer cell fractions were analysed. For each purified cell fraction mean purity values were calculated from the FACS data (table 3.2). MiniMACS beads directly conjugated to CD14 were first used to isolate monocytes as these cells express Fc-receptors and are generally adherent so could non-specifically bind to antibody coated beads for other cell types. Monocytes contained a mean frequency (11 samples) of 0.3% contaminating T cells (CD3 positive) and only 1.2% CD19 positive B lymphocytes and 1.6% CD16 positive NK cells. CD4 conjugated beads were then used to isolate CD4 lymphocytes (as well as CD4 expressing DCs). CD4 selected cells were

Table 3.2 : Purity of Cell Fractions Isolated with the MiniMACS System by FACS Analysis.

	% Cells	% Lymphocytes			% Cells
Selected Cell Subset	**CD3[b]**	**CD4[b]**	**CD8[b]**	**CD19[b]**	**CD16[b]**
CD8 + T Lymphocytes[c]	95 (92.1-99.2)[d]	1.6 (0.3-2.1)	93 (91.5-98.2)	0.2 (0.1-0.4)	1.6 (0.3-3.7)
CD4 + T Lymphocytes[c]	97.6 (96.0-99.3)	90.4 (86.4-98.8)	1.6 (0.4-4.2)	0.1 (0.0-0.2)	1.0 (0.3-2.2)
Monocytes[e]	0.3	ND[a]	ND	1.2	0.2
Natural Killer Cells[e]	3.6	0	0	0	93.5

a ND= Not Done; b CD3, CD4, CD8, CD19, CD16= Monoclonal antibodies preconjugated to fluorescent dyes used for FACS analysis.
c CD4 and CD8 lymphocytes: Data from 11 samples, 5 negative controls and 6 from separated HIV positive samples.
d Values in brackets are ranges of cell purity for the 11 samples analysed.
e Natural Killer cells and monocytes: Data from one HIV-positive sample

97.6% CD3 positive and 90.4% CD4 positive. However there were only 1.6% contaminating CD8 cells, 0.1% B Cells and 1.0% NK cells. The percentage of CD4 expressing cells may be higher but masked by bound CD4 MiniMACS beads. In all individuals but three CD56 coated beads (Immunotech) were used to deplete NK cells and reduce potential contamination to the CD8 lymphocyte fraction. In the remaining three individuals a sample of CD56 coated MiniMACS beads were used to positively select NK cells for FACS analysis and PCR. The NK cell fractions were 93.5% CD16 positive and only 3.6 % CD3 positive. Finally anti-CD8 coated MACS beads were used to positively select CD8 lymphocytes from the CD14, CD4, CD56 depleted cell fraction. These cells were 95% CD3 positive (mean 11 samples) and at least 93% CD8 positive. Contaminating CD4 cells and NK cells were only 1.6% of the cell population.

As well as allowing direct FACS analysis of cell subsets the miniMACs method provided a rapid system which could be used to isolate cells. The time taken to separate cells proved important in RNA work carried out using the purified cells (chapter 4).

3.10 Detection of HIV-1 DNA in different cell subsets using nested-PCR

Once the cell fraction purity was determined nucleic acid was extracted and proviral DNA was quantified in each cell type using a limiting dilution and nested-PCR approach (Simmonds *et al.*, 1990b). The quantitation was performed using primers corresponding to the *gag* gene and the V3 region. A nested PCR approach

was used because this method can detect one single molecule of target DNA, and allow quantitation by dilution of DNA to an end-point. PCR product was visualised on a 2% agarose gel containing ethidium bromide. Using cell counts from each sample and the number of copies of provirus detected it was possible to express the results as proviral copies per million cells. All separations, extractions, and amplifications were carried out with parallel samples of mononuclear cells isolated from buffy coat leucocytes derived from HIV-1 negative blood to serve as negative controls. Serial dilutions of cells from HXB2 cultured cells with a known PCR cut off were used as positive controls for extractions and PCR reactions.

HIV-1 DNA could be detected in CD4 T lymphocytes, CD8 T lymphocytes, monocytes, DCs and NK cells (Tables 3.3 and 3.4). HIV DNA was never detected in the negative controls or purified B cells. For negative samples the cut-off for the assay depended on the number of cells isolated and available for extraction. Mean CD4 counts taken over the previous six months were used as a marker of immunosuppression and progression to AIDS. HIV DNA was detected in the CD4-positive cell subset of 17 of the 19 individuals with CD4 counts less than 200, and in 10 of the 11 individuals with CD4 counts greater than 200. Where the E-rosetting technique was used CD4 lymphocytes were isolated and were found to be infected with HIV in 8 of the 10 individuals with mean CD4 counts less than 200/μl and in 6 of the individuals with CD4 counts greater than 200 (Table 3.3). When the miniMACS method was used to isolate CD4 cells the cell fraction contained both CD4 lymphocytes and CD4 expressing populations of DCs. Using this method

Table 3.3 : Frequency of HIV-1 Infection in Cell Subsets Isolated by E-Rosetting and MAb-coated Magnetic Beads.

		FREQUENCY INFECTION (Proviral copies/10^6 cells)				
Patient	**Mean CD4 Count/μl**	**CD4+ T Cells**	**CD8+ T Cells**	**Monocytes**	**B cells**	**Dendritic Cells**
P22	0	>4882	110	40	<10	60
P26	0.5	>483	58	4	<10	200
P5	4	4873	4	<4	<7.2	180
P29	13	<10	<20	<100	<100	70
P24	14	44	34	<10	<20	23
P31	35	98	46	NR[a]	NR	NR
P27	54	85	6	4	<1	<10
P7	63	208	24	20	<2	60
P3	80	39	2	<1 [b]	<2	20
P28	143	<2	<10	<10	<100	340
P1	228	400	400	<1	<1	700
P6	236	40	1	<0.25	<2.5	6
P30	279	2	<20	<1	<10	1099
P23	305	117	<10	<10	<10	<10
P25	576	<100	<10	<100	<10	<50
P4	860	11	0.5	<0.5	<0.08	40
P2	938	2	<0.05	<0.8	<1	<1

a NR= no result; b For negative samples the cut off for assay depended on the number of cells extracted.

Table 3.4 : Frequency of HIV-1 Infection in Cell Subsets Isolated by MiniMACS and Dynabeads.

		FREQUENCY INFECTION (Proviral copies/10^6 cells)				
Patient	**Mean CD4 Count/ml**	**CD4+ T Cells**	**CD8+ T Cells**	**Monocytes**	**NK Cells**	**PBMC**
S09	3	>100	>100	<1 [b]	NR[c]	NR
S08	8.3	>1000	>1000	<1	NR	NR
S15	10	141	17	2225	NR	12
S07	22	>100	>1000	<1	NR	NR
S24	23	1115	261	3	47	723
S06	56	10	8.7	<1	NR	49
S04	73	128	1400	<1	NR	41
S11	85	1620	19	71	NR	NR
S19	90	498	3	19	NR	333
S26	184	25	25	<10	NR	NR
S14	354	3817	65	5	NR	NR
S21	386	568	435	<0.7	NR	NR
S05	406	1.1	85	<1	NR	1.1
S22	418	50	25	286	86	716
S25	420	135	<55	<160	61	NR
Anon 1[c]	NR	<2	<5	<10	NR	NR
Anon 2	NR	85	23	13	NR	56

a NR= no result; b For negative samples the cut off for assay depended on the number of cells extracted. c Anon= Blind study individuals separated using dynabeads.

HIV DNA was detected in all the individuals studied, 10 with CD4 counts less than 200 and 5 with CD4 counts greater than 200. CD4 lymphocytes were infected at all stages of disease progression. The mean level (1071 proviral copies/10^6 cells) was higher in individuals with CD4 counts less than 200 than for those with higher counts (82 copies/10^6 cells) (table 3.5). Infection was detected in monocytes of seven individuals with CD4 counts from <1 to 71 and in two individuals with CD4 counts of 286 and 382/μl. The mean frequency of infection in those individuals with CD4 counts <200 was 184 proviral copies/10^6 cells and 27 copies/10^6 cells in individuals with higher CD4 counts. Dendritic cell infection ranged from <1 to 1099 proviral copies/10^6 cells in individuals at all stages of disease progression. The frequency of infection of dendritic cells did not correlate with CD4 count ($p=0.490$ Wilcoxon signed ranks test), and the individual with 1099 copies/10^6 DCs had a CD4 count of 279/μl. HIV DNA was detected in the CD8 lymphocytes of 23 of the 34 individuals studied. These HIV positive CD8 lymphocytes included 6 individuals with CD4 counts greater than 200. The mean frequency of infection in individuals with CD4 counts greater than 200 was 86 compared to 206 proviral copies/10^6 cells in individuals with lower CD4 counts. In the three individuals where NK cells fractions were extracted and PCR reactions carried out, HIV DNA was detected in 3 of the cell isolates, from individuals with CD4 counts of 400 and 366/μl.

Table 3.5 : Mean Cell Counts, Frequency of infection, and Distribution of infection in PBMCs for HIV seropositive individuals with CD4 counts/μl of greater than and less than 200.

	CD4 Count >200/μl [a]	CD4 Count <200/μl [b]
Mean CD8 Counts	1051	625
CD4 Loss[e]	5.5	32
CD8 Loss [e]	-1.4	19
Frequency infection CD4 T cells [c/f]	82	1071
Frequency infection CD4 T cells/DCs [c/g]	1109	500
Frequency infection CD8 T cells [c]	80	238
Frequency infection monocytes [c]	29	95
Frequency infection DCs [c]	264	106
Distribution HIV in CD4 T cells [d]	54	21
Distribution HIV in CD4 T Cells/DCs [d]	40	8
Distribution HIV in CD8 T cells [d]	31	59
Distribution HIV in monocytes [c]	7	4
Distribution HIV in DCs [c]	18	23
Distribution HIV in NKs [c]	3	/

a For individuals with CD4 counts <200 the mean values are for 18 individuals. b The mean values for individuals with CD4 counts >200 were calculated using data from 14 individuals. c Frequencies of infection are in proviral copies/10^6 cells. d Distribution values in each cell subset are given as a % of the total proviral load in the total PBMCs of each individual. e CD4 and CD8 cell loss values are a percentage loss from a cell count taken 12 months prior to the sample. f CD4 infection=CD4 cells separated by E-rosetting method, g CD4/DCs=CD4+ cells isolated using MiniMACS system. All calculations exclude individuals receiving anti-retroviral therapy.

CD4 and CD8 cells were separated in an independent laboratory from PBMCs of two control HIV negative individuals and two HIV seropositive individuals. These samples were labelled anonymously and were analysed for the presence of HIV DNA. HIV was detected in both the CD4 and CD8 cells isolated from the HIV seropositive individuals but no HIV DNA was detected in the negative control samples.

3.11 Analysis of quantification results

To investigate the relationship between the frequency of HIV infection of CD4 T lymphocytes and CD4-positive cells containing DCs with disease progression, mean CD4 counts/μl were plotted against the frequency of infection of CD4 T lymphocytes (Figure 3.1) and CD4 cells isolated using the miniMACS system (Figure 3.2). Although there was a wide variation in CD4 lymphocyte infection ranging from 1 to almost 10000 copies/10^6 cells an inverse correlation with mean CD4 number was observed ($p = < 0.001$) No correlation was evident between mean CD4 counts and the frequency of infection of CD4-positive cells isolated without prior separation of T and non-T cells ($p = 0.463$). The frequency of infected CD8 lymphocytes was also variable ranging from 1 to 100 copies/10^6 cells (Figure 3.3), with an observable tendency for an increase in the frequency of HIV infection with declining CD4 count ($p = 0.03$) .

The frequencies of HIV infection of CD4 and CD8 lymphocytes (HIV copies/10^6 cells) were then plotted (Figure 3.4). There was a good correlation

Figure 3.1 : The Frequency of infection of CD4 lymphocytes (log_{10} copies/10^6 cells) isolated by E-rosetting followed by positive selection with anti-CD4 coated magnetic beads (Immunotech) vs the mean CD4 counts/µl whole blood calculated over a six month period.

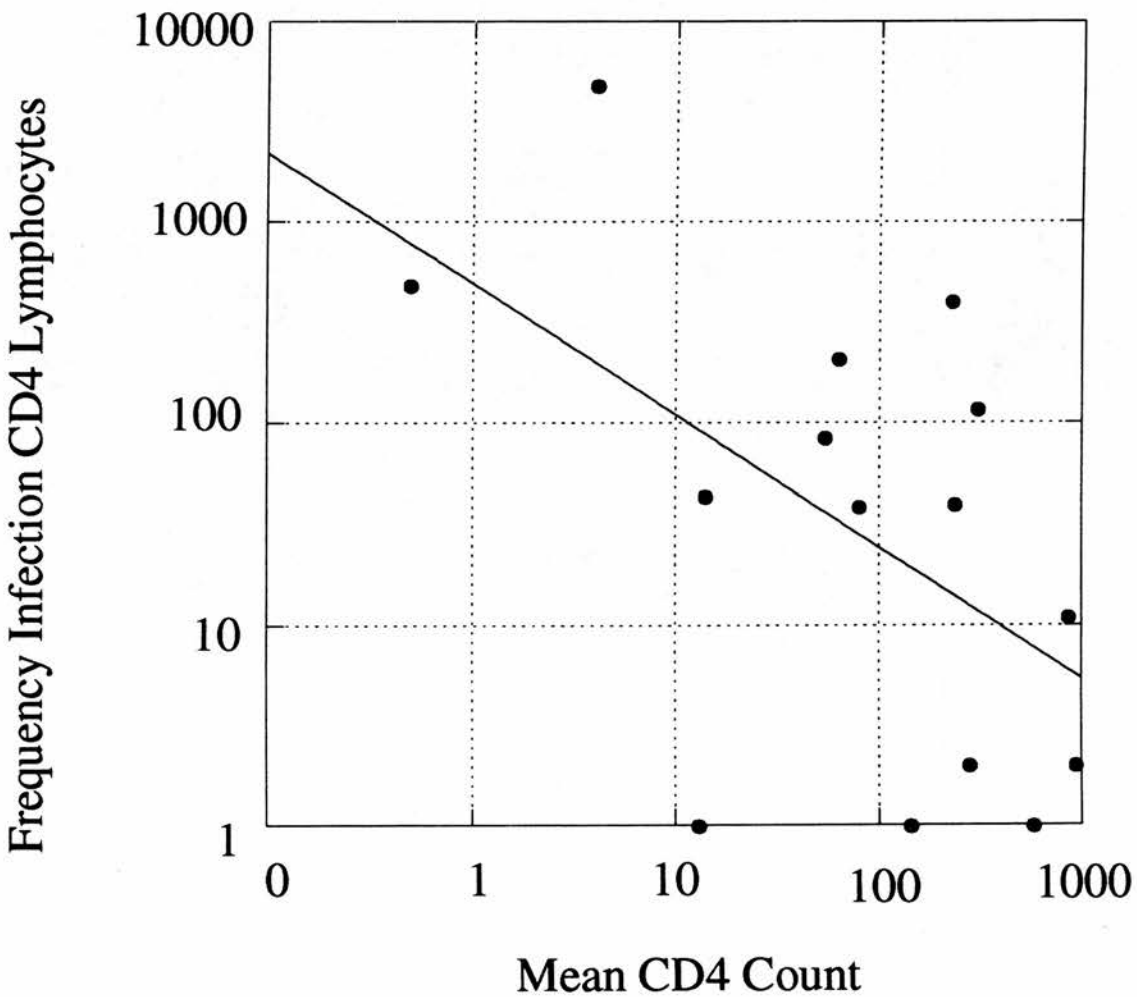

Frequency Infection CD4 Lymphocytes
10000
1000
100
10
1
0
1
10
100
1000
Mean CD4 Count

Figure 3.2 : The Frequency of infection of CD4-positive cells including CD4 T cells and Dendritic cells (log_{10} copies/10^6 cells) isolated by miniMACS vs the mean CD4 counts/µl whole blood calculated over a six month period.

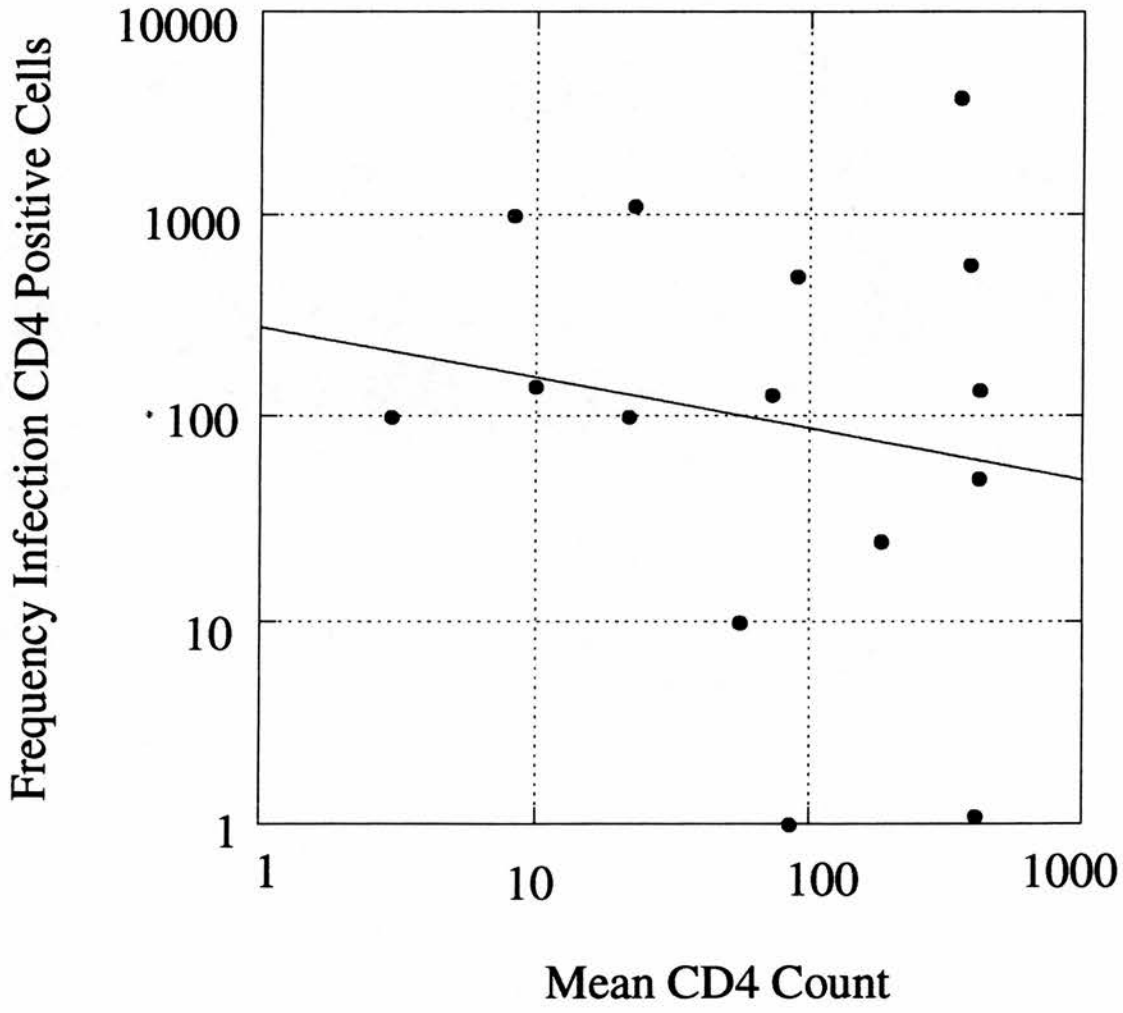

10000
1000
100
10
1
1
10
100
1000
Frequency Infection CD4 Positive Cells
Mean CD4 Count

Figure 3.3 : The Frequency of infection of CD8 lymphocytes (log_{10} copies/10^6 cells) isolated by E-rosetting followed by positive selection with anti-CD8 coated magnetic beads and by miniMACS vs the mean CD4 counts/µl whole blood calculated over a six month period.

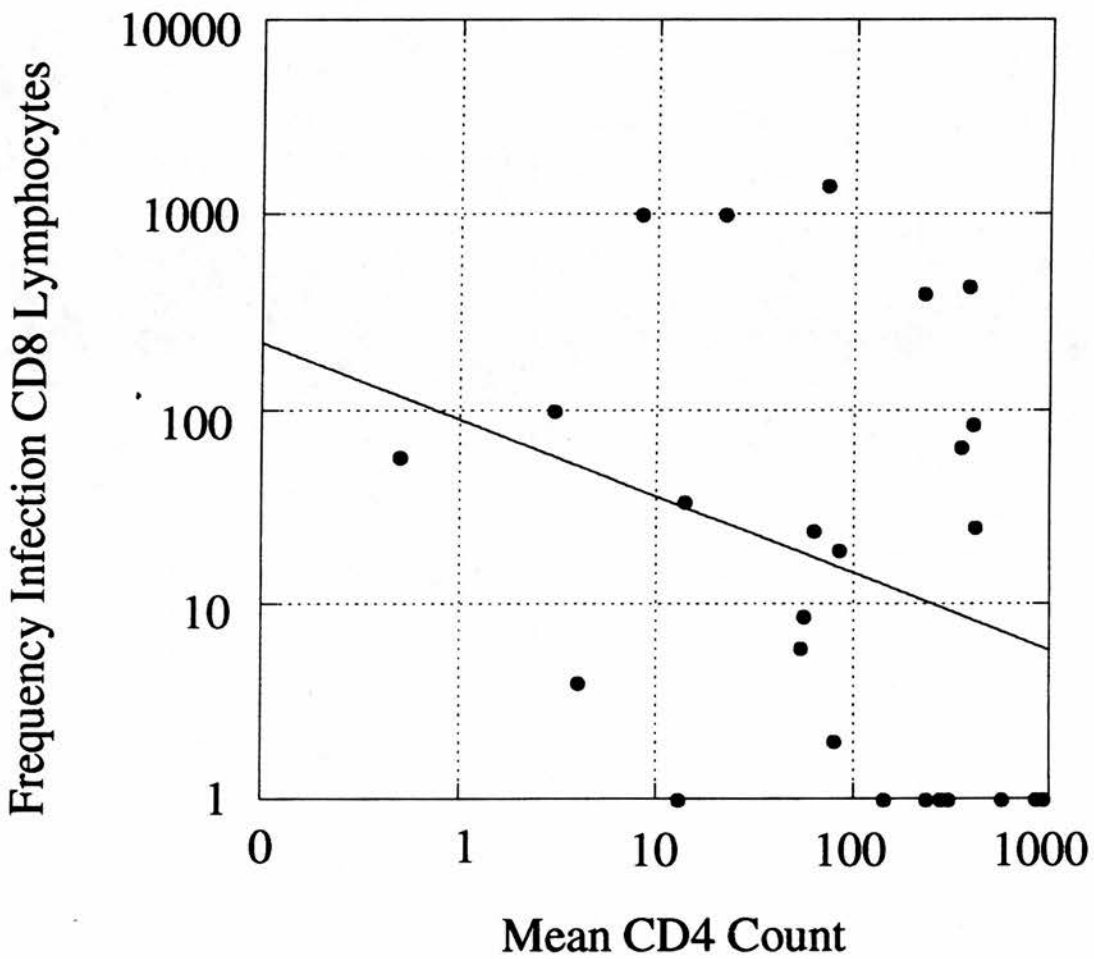
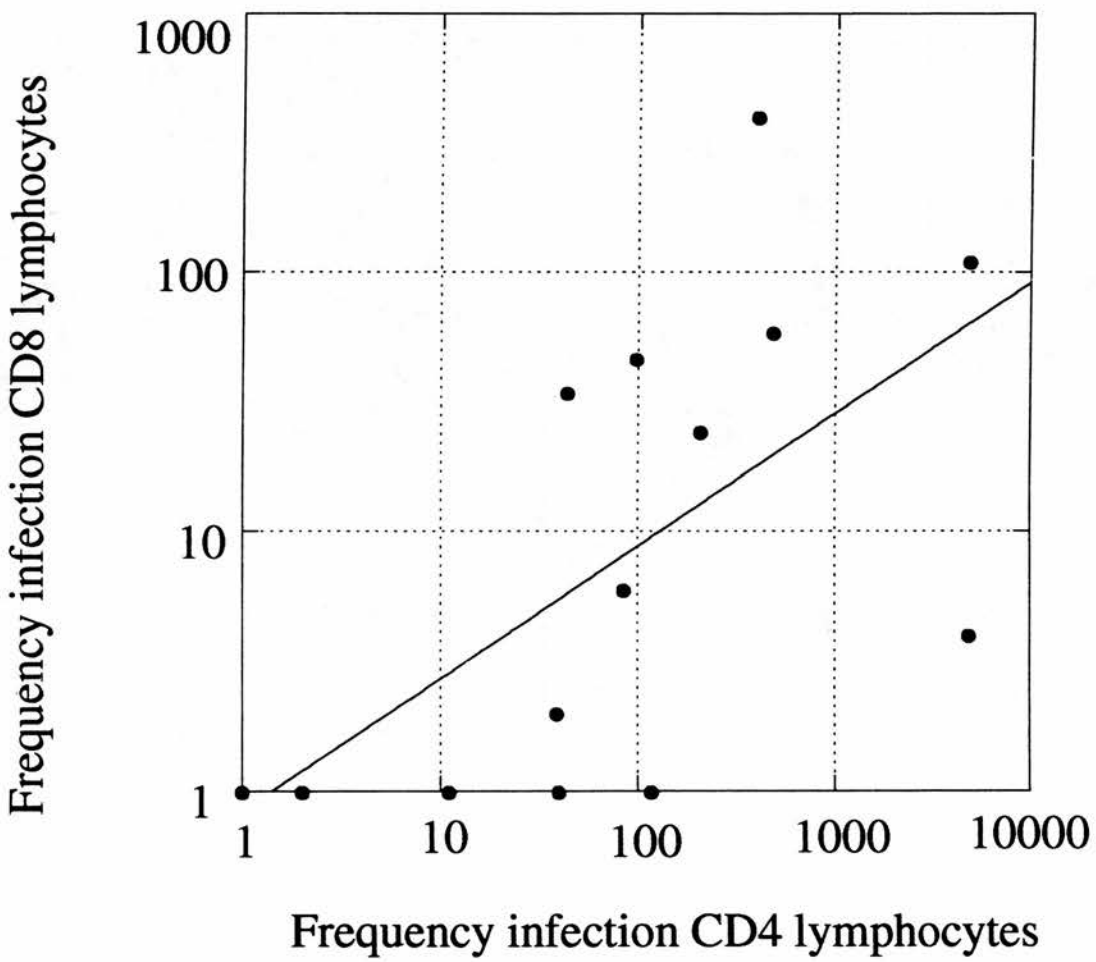

Frequency Infection CD8 Lymphocytes
10000
1000
100
10
1
0
1
10
100
1000
Mean CD4 Count

Figure 3.4 : The Frequency of infection of CD4 lymphocytes (log_{10} copies/10^6 cells) isolated by E-rosetting followed by positive selection with anti-CD4 coated magnetic beads (Immunotech) vs the frequency of infection of CD8 lymphocytes (log_{10} copies/10^6 cells).


1000
100
10
1
1
10
100
1000
10000
Frequency infection CD8 lymphocytes
Frequency infection CD4 lymphocytes

between the infection of CD4 T lymphocytes and CD8 T lymphocytes ($p=0.002$). As the frequency of HIV infection in CD4 lymphocytes increases the frequency of infection of CD8 lymphocytes increases. Less of a correlation was evident when the frequency of CD8 infection was plotted against CD4 positive cell infection isolated using the miniMACS method ($p=o.312$) (including CD4 lymphocytes and DCs).

A total of 14 samples contained no detectable infection in monocytes, yet were obtained from individuals with a wide range of CD4 counts. Apart from the two individuals receiving anti-retroviral therapy the provirus load in monocytes was less than 100 proviral copies/10^6 cells. No correlation between disease progression and monocyte infection was apparent ($p=0.139$) (figure 3.5). The frequency of DC infection was also plotted against mean CD4 count (figure 3.6). The level of infection was generally higher than that in the monocyte population and there were fewer negatives. The frequency of infection of DCs did not correlate with CD4 counts (0.).

The absolute cell counts for each cell fraction and the frequencies of infection were combined to determine the contribution of HIV-1 DNA from each cell subset to the proviral load in total PBMCs. Absolute cell counts were used to calculate the proviral load of each cell subset per µl of whole blood. The contribution of each cell subset to the overall proviral load in PBMCs (% total load in PBMCs) were then illustrated as bar graphs with individual patients plotted along the X-axis in ascending order according to mean CD4 counts (cells/μl). Separate

Figure 3.5 : The Frequency of infection of monocytes (log_{10} copies/10^6 cells) isolated by positive selection with anti-CD14 coated magnetic beads (Immunotech and miniMACS) vs the mean CD4 count calculated over a six month period.

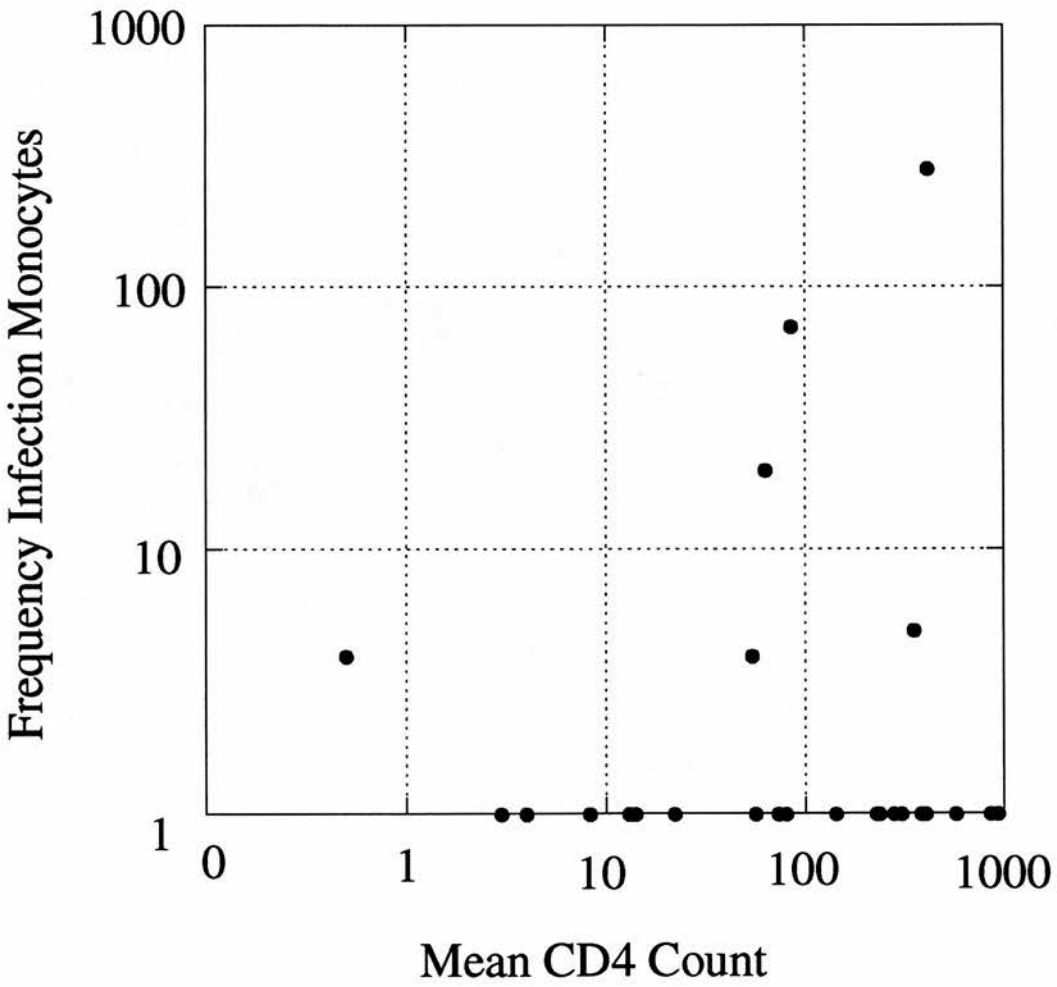
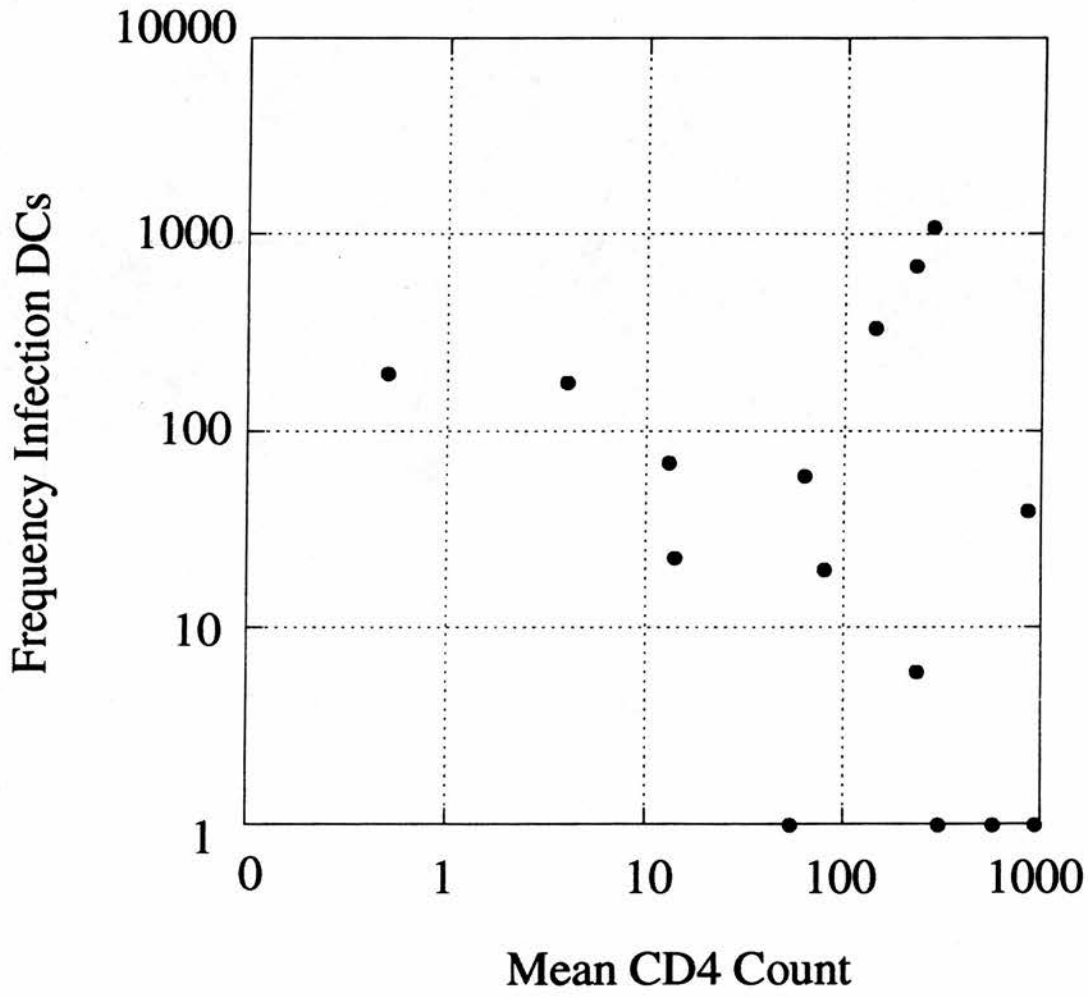

1000
100
10
1
Frequency Infection Monocytes
0
1
10
100
1000
Mean CD4 Count

Figure 3.6 : The Frequency of infection of Dendritic cells (log_{10} copies/10^6 cells) vs the mean CD4 count over a six month period.


10000
1000
100
10
1
0
1
10
100
1000
Frequency Infection DCs
Mean CD4 Count

graphs were plotted for samples separated by the E-rosetting method (figure 3.7) and those using the miniMACS technique (figure 3.8). Individual p25 appears blank because a low level of virus was detectable in the total PBMCs it was not possible to detect virus in any of the individual cell subsets. Individuals S15, S19, S24, S25, and S26 were all recieving anti-retroviral therapy. Looking at both figures it is evident that there appears to be very little correlation between the distribution of monocyte and dendritic cell infection with disease progression ($p=0.139$ and 0.09 respectively). However as would be expected as the CD4 count decreases so does the contribution of provirus from CD4 lymphocytes to the total viral load in PBMCs. This also true for CD4 positive cells isolated using miniMACS beads. This cell fraction contained DCs but because these cells are a minor population ($<1\%$ of PBMCs) their presence may not affect the overall contribution of CD4 lymphocytes.

It is also evident that as the disease progresses the contribution of CD8 lymphocytes increases, so in individuals with lower CD4 counts, CD8 lymphocytes become a major reservoir of HIV infection in the peripheral blood. This increase in the relative viral load in CD8 lymphocytes of late stage individuals is especially clear in S8, P24, S9 and S7 where the individuals had CD4 counts of less than $20/\mu l$ and the CD8 lymphocytes contributed almost 100% of the total HIV population in PBMCs.

Figure 3.7 : The relative distribution of different cell subsets isolated by E-rosetting and positive selection with monoclonal antibody coated beads (Immunotech) to proviral load in peripheral blood mononuclear cells.

CD4 = CD4 lymphocytes; CD8 = CD8 lymphocytes; DC = Dendritic cells; mono = Monocytes.

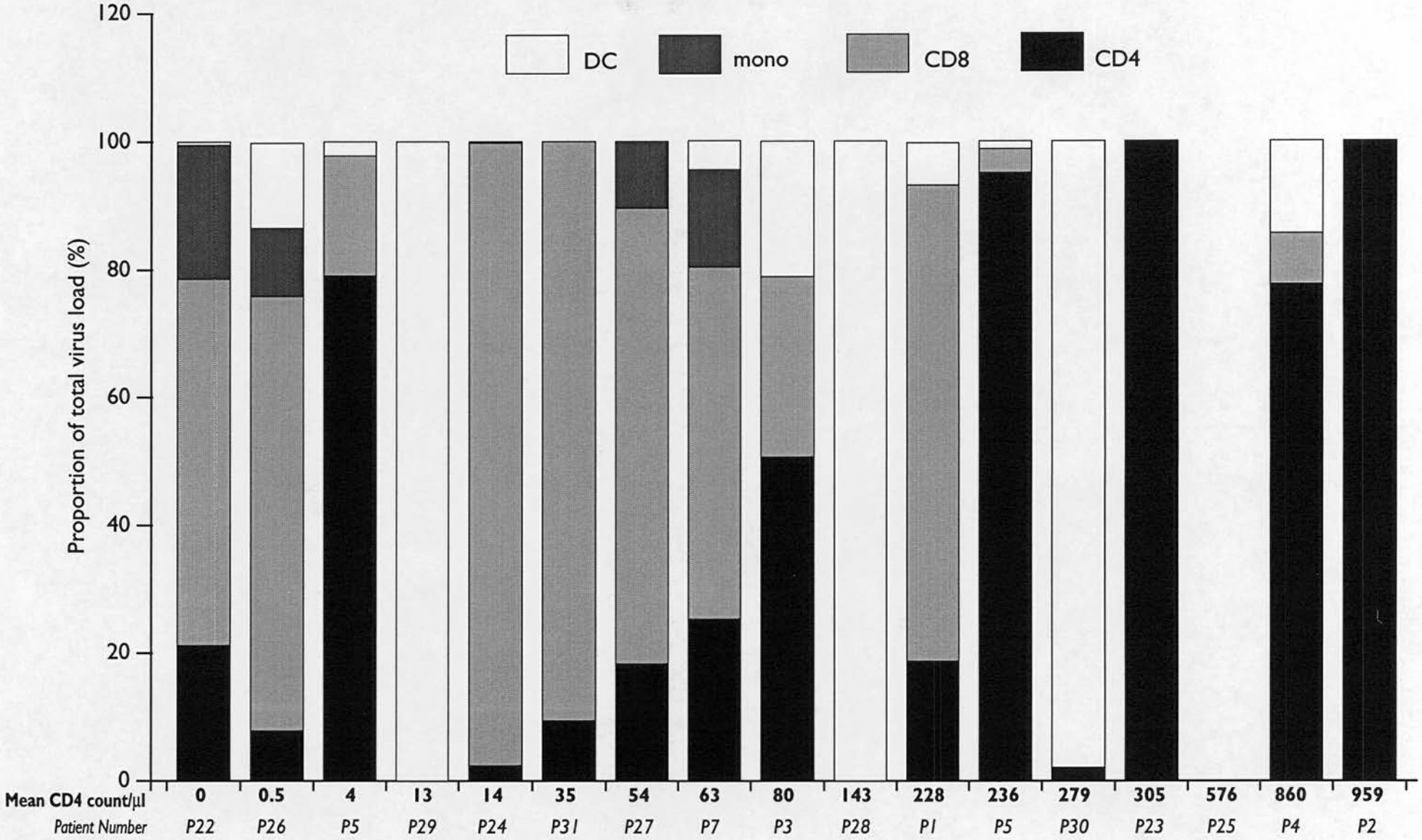
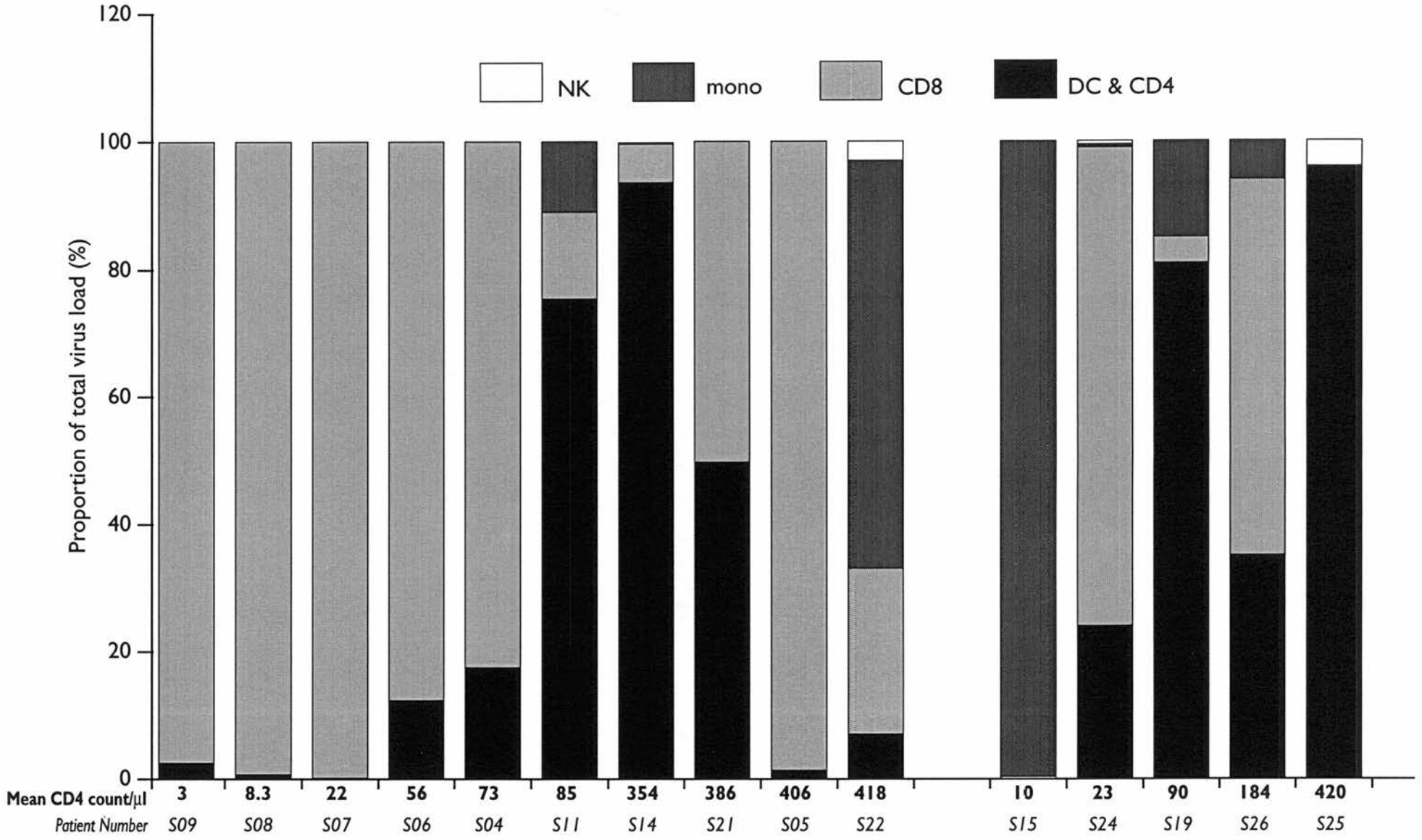

120
100
80
60
40
20
0
Proportion of total virus load (%)
DC
mono
CD8
CD4
Mean CD4 count/µl
0
0.5
4
13
14
35
54
63
80
143
228
236
279
305
576
860
959
Patient Number
P22
P26
P5
P29
P24
P31
P27
P7
P3
P28
P1
P5
P30
P23
P25
P4
P2

Figure 3.8 : The relative distribution of different cell subsets isolated by miniMACS to proviral load in peripheral blood mononuclear cells.

CD4 = CD4 lymphocytes and dendritic cells; CD8 = CD8 lymphocytes; NK = Natural killer cells; mono = Monocytes.

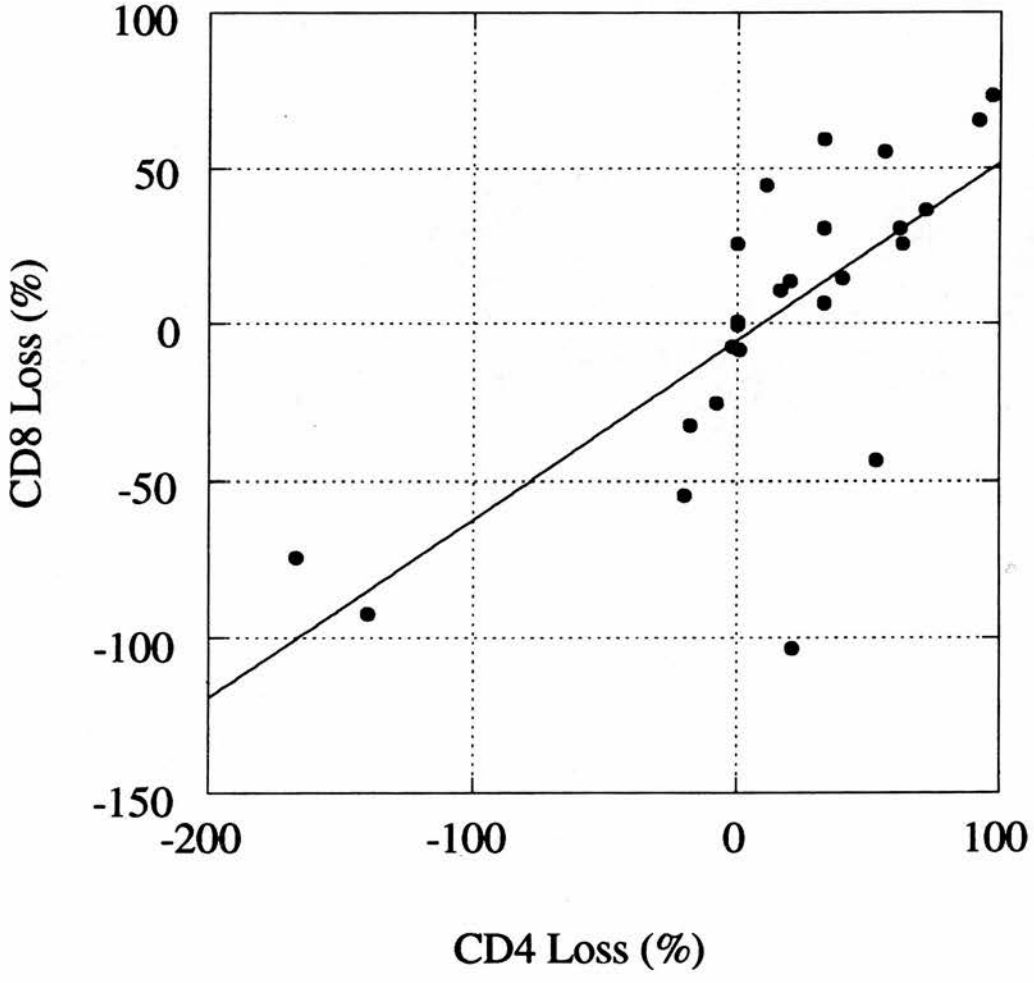

120
100
80
60
40
20
0
Proportion of total virus load (%)
NK
mono
CD8
DC & CD4
Mean CD4 count/µl
3
8.3
22
56
73
85
354
386
406
418
10
23
90
184
420
Patient Number
S09
S08
S07
S06
S04
S11
S14
S21
S05
S22
S15
S24
S19
S26
S25

3.12 CD4 and CD8 cell loss with progression to AIDS

CD4 and CD8 cell loss over a twelve month period prior to the study were calculated for each of the 34 individuals. CD4 loss and CD8 cell loss were plotted as a percentage depletion in that cell type (figure 3.10). Therefore a positive value represents cell loss, a value of zero means there was no change in the cell count and a negative value represents an increase in the cell count for either CD4 or CD8 lymphocytes. From the graph it is evident that in the majority of individuals there was a decrease in both the number of CD4 cells and CD8 lymphocytes. Five individuals showed an increase in CD4 lymphocyte numbers and nine showed an increase in CD8 cell numbers, of which two were receiving anti-retroviral therapy. It is also evident that the level of CD4 and CD8 loss correlate, that is where there is a high CD4 loss CD8 loss is also high.

In order to investigate cell loss amongst memory and naive CD4 and CD8 lymphocytes, cells from 50 individuals were stained with monoclonal antibodies directed against CD4, CD8 , CD45RA and CD45RO. These values were plotted against CD4 counts (figure 3.11). In both cell subsets, CD4 and CD8 lymphocytes, naive cells were lost in preference to memory cells. The majority of the cells expressed CD45 RA, a naive cell marker, in individuals with CD4 counts of greater than 500. However as CD4 counts fall to 200-500 cell per μl there is a sharp decrease in the number of naive cells so the memory cell subset become the major cell population in both CD4 and CD8 lymphocytes.

Figure 3.9 : CD8 lymphocyte loss over a 12 month period (%) vs CD4 lymphocyte loss over a 12 month period (%). Cell loss calculated from absolute cell counts determined by FACS analysis.


100
50
0
-50
-100
-150
-200
-100
0
100
CD8 Loss (%)
CD4 Loss (%)

3.13 Effect of PHA stimulation on CD4 and CD8 lymphocytes

In a recent report it has been suggested that anti-CD3 or phytohaemagluttin (PHA) may up regulate CD4 expression on the surface of CD8 T lymphocytes. In order to investigate whether CD4 up regulation on CD8 T cells may be a possible mechanism of infection of this cell subset, purified cells were FACS analysed for CD3, CD4, and CD8. These cells were then stimulated with PHA, a plant derived T cell mitogen. In all analysed cells the percentage of CD3 cells was at least 97.4% (table 3.6). Before stimulation 1.6% of CD4 cells expressed both CD4 and CD8 and after stimulation this increased to 7.5%. In the purified CD8 lymphocyte population the number of CD4, CD8 double positive cells increased from 0.4% to 21.7% after stimulation. There was no increase in CD3-positive, CD4-positive, CD8-negative lymphocytes in the stimulated CD8 cells, therefore it is possible that CD4 is up-regulated on the surface of CD8 cells when stimulated with PHA.

DISCUSSION

3.14 Infection of a wide range of cell types within PBMCs in vivo by HIV

This study documents the infection of a wide range of different cell types within the peripheral blood of seropositive individuals by HIV. HIV DNA sequences were detected in CD4 T lymphocytes, monocytes, peripheral blood dendritic cells, CD8 T lymphocytes and natural killer cells. There was remarkable individual variation in the frequencies of infected cells within each cell subset.

Table 3.6 : Efect of PHA Stimulation on the level of T Cells Expressing both CD4 and CD8 antigens.

Selected Cell Subset	Culture Status	% Cells CD3+ [b]	CD4/CD8[b] Double Positive Lymphocytes
CD4+ T Lymphocytes	Freshly isolated cells (no stimulation in culture)	97.6 %	1.6 %
CD8+ T Lymphocytes	Freshly isolated cells (no stimulation in culture)	97.4 %	0.4 %
CD4+T Lymphocytes	Cells stimulated with PHA[a] in culture for 7 days	98.9 %	7.5 %
CD8+ T Lymphocytes	Cells stimulated with PHA in culture for 7 days	98.5 %	21.7 %

a PHA= Phytohaemaglutinin a T cell mitogen.
b CD3, CD4 and CD8= Monoclonal antibodies pe-conjugated to fluorescent dyes used for FACS analysis.

As previously described HIV infection of CD4 lymphocytes were detected at all stages of disease progression (Schnittman *et al.,* 1989). HIV-1 DNA was only detected in the monocyte fraction in 7 of the 29 treatment naive individuals and the level of infection detected was consistently low except in one individual undergoing therapy. This is consistent with previous reports which suggested a low level of monocyte infection *in vivo* and the requirement for differentiation into macrophages for HIV infection (Schnittman *et al.,* 1989; Innocenti *et al.,* 1992; Bagasra *et al.,* 1993; Hsia *et al.,* 1995). In the small study undertaken we were able to detect HIV-1 infection of natural killer cells isolated directly from the peripheral blood of infected individuals. This was a novel finding although purified NK cells are infectable *in vitro* and this may explain why NK cell numbers and function decrease with disease progression (Chehimi *et al.,* 1991; Scottalgara *et al.,* 1993).

Previous reports of infection of DCs in vivo are contradictory. For example, Hsia *et al* were not able to detect any HIV-1 DNA in 10^4 or 10^5 DCs isolated from the PBMCs of seropositive individuals (Hsia *et al.,* 1995), whereas Patterson *et al* detected HIV provirus by PCR in lymphocytes and DCs of all the patients investigated and found that in each case the viral load was similar for both cell fractions (Patterson *et al.,* 1994). This study would support the latter finding as HIV infection of peripheral blood dendritic cells was detected at all stages of disease progression. No correlation was observed between the frequency of DC infection and disease progression. However, it was difficult to accurately determine the

frequency of infection of DCs as there was no way to determine how effective the DC isolation was in each case and how many of the DCs were selected in the E-rosette positive fraction. Any variation in results from investigating infection of DCs may be due to different methods used to purify the cells. Weissman *et al* isolated three populations of DCs from the peripheral blood using three different purification methods and found only one of these isolated DC subsets to be infectable with HIV (Weissman *et al.,* 1995).

A surprising finding in this study was the frequent infection of CD8 lymphocytes in symptomatic and pre-AIDS individuals. HIV DNA was detected in the CD8 lymphocytes of 23 of the 34 individuals studied. These HIV positive CD8 lymphocytes included 6 individuals with CD4 counts greater than 200 per μl. It was not possible for contamination of the CD8 population with another cell type to account for the levels of HIV-1 DNA detected, for example in S04 1400 copies HIV/10^6 CD8 cells were detected compared to only 128 copies/10^6 CD4 cells and from FACS analysis CD4 contamination of CD8 cells was only 1.6%. These findings are inconsistent with previous reports which found that CD8 cells were uninfected *in vivo* (Psallidopoulos *et al.,* 1989; Schnittman *et al.,* 1989). It is difficult to identify the reason for the discrepancy, although a relatively insensitive PCR was used in both of these earlier studies that may have been incapable of detecting the low levels of infection found in some of the study group. However, Semenzato *et al* recently been reported that CD8 lymphocytes recovered from the lung tissue of AIDS patients show similar frequencies of infection to those in

PBMCs reported here (Semenzato *et al.,* 1995). Infection of peripheral blood CD8 cells has been reported with SIV and CD8 lymphocytes are susceptible to HIV-1 infection in vitro in the presence of CD4 T lymphocytes. De Maria et al demonstrated that CD8-positive, CD4-negative lymphocyte lines derived from infected individuals express HIV proteins and generate reverse transcriptase activity. Infection was confirmed at a single cell level by immunoelectron microscopy and two colour immunohistochemistry. The basic requirements for HIV infection of CD8 lymphocytes was the presence of CD4 cells. PCR of sorted cells revealed that the CD8 cells harboured HIV-1 proviral DNA and stimulation of these cells resulted in virus replication and infection of CD4 cells (De Maria *et al.,* 1991).

Following publication of the findings of this study Sleasman *et al* reported that in children and neonates HIV-1 DNA could be detected in CD4 but not CD8 lymphocytes (Sleasman *et al.,* 1996). However, only a small number of individuals were studied, all of whom had CD4 counts greater than 200. A single round PCR method was used and the maximum number of cells analysed was only 10^4. In most pre-AIDS cases in this study HIV DNA would not have been detected in CD8 lymphocytes using the PCR method described. Most recently it has been reported that proviral sequences were detected in CD8 lymphocytes from 10 of 12 HIV infected individuals (Flamand *et al.,* 1997). This study highlights HIV infection of CD8 lymphocytes *in vivo* and suggests that reactive CTLs recruited to kill HIV infected cells could, as a consequence of specific antigen activation, express CD4 and become targets for HIV infection.

In general the frequency of CD4 lymphocyte and CD8 lymphocyte infection increased with disease progression. However there appeared to be very little correlation between the level of DC or monocyte infection with mean CD4 counts. In order to get a clearer picture of HIV infection of different cell subsets with disease progression the level of infection was expressed as a percentage of the total HIV load in PBMCs. This analysis revealed that early in infection CD4 lymphocytes are the major cell type in the peripheral blood infected with HIV. However as the disease progresses and the CD4 count decreases, CD8 lymphocytes become a major reservoir of HIV in the peripheral blood. The significance of widespread infection of CD4 negative, CD8 positive lymphocytes in late stage disease remains to be determined and will be addressed in subsequent chapters. One of the contributing factors of the high frequency is the loss of CD4 T cells which effectively removes this population from circulation. The substantial increase in CD8 T cell infection on disease progression may possibly be related to the phenomenon by which HIV spreads to non-lymphoid tissue during the later stage of disease, associated with the loss of immune control.

3.15 Possible mechanisms of HIV infection of CD4 negative cells.

If CD8 lymphocytes in the peripheral blood are infected then the mechanism of HIV attachment and entry remain unclear. Early in T cell ontogeny, thymocytes express both CD4 and CD8. As these T cells further differentiate, they loose either CD4 or CD8 expression, and coincident with this acquire their functional

capabilities. Occasional lymphocytes co-expressing the CD4 and CD8 antigens are present in normal human blood peripheral lymphocytes (Lundberg *et al.*, 1995). Although these cells constitute less than 3% of the total circulating T cell population in uninfected individuals, an increase in lymphocytes expressing both CD4 and CD8 antigens to 43% of total lymphocytes has been reported in an HIV infected patient (Ribrag *et al.*, 1993). Infection of these double positives CD8 lymphocyte precursors may be a possible mechanism for CD8 infection with HIV using the CD4 receptor. To investigate the levels of CD4 CD8 double positive lymphocytes in circulation in HIV-1 positive individuals and negative controls FACS analysis was carried out using a panel of anti-CD3, CD4, and CD8 monoclonal antibodies. In the HIV-1 negative individuals studied only a low level of lymphocytes co-expressing CD4 and CD8 were detected (mean value 0.3%). No increase in CD4 CD8 T cells was noted in PBMCs isolated from 40 HIV seropositive individuals (Mason, 1997). This low level of CD4 expressing lymphocytes in circulation would be unlikely to account for the level of infection detected, and in any case CD4 cells were depleted first in all separation methods so any immature double positive lymphocytes would be purified with the CD4 positive cells. It is possible however, that HIV infects double positive precursors in the thymus. As these T cells further differentiate they may loose CD4 expression and in this study these cells would be isolated in the CD3 positive, CD4 negative, CD8 positive cell fraction. Thus the peripheral CD8 cells we observed to be infected with HIV could have been infected within the thymus while expressing CD4 during normal selection.

Although mature CD8 lymphocytes do not express CD4, in vitro infection of CD8 lymphocytes with HIV-1 has been achieved by co-culture with HIV-1 infected CD4 cells (De Maria *et al.,* 1991). It is possible that the interaction between CD4 and CD8 cells occuring in vivo as part of the aquired immune response may transmit infection to CD8 lymphocytes. This would involve infection via cell-to-cell contact, the most common opportunity for CD8 T lymphocytes to come into close contact with HIV-1 infected CD4 cells *in vivo* is during their lysis in lymphoid organs.

Another possible mechanism of CD8 infection using CD4 could involve transient up regulation of CD4 expression on CD8 lymphocytes during antigenic stimulation of the cell. This is supported by the finding that mature CD8 lymphocytes do not express CD4 at the cell surface but CD4 mRNA can still be detected by RT-PCR in CD8 cells isolated both from PBMCs and cells collected by bronchiolar lavage (Semenzato *et al.,* 1995). In this study we were able to show that PHA up-regulates CD4 expression on CD8 cells *in vitro* however this was a very artificial way of examining T cell stimulation and may not reflect events *in vivo*. A recent study reported that stimulation of CD8 lymphocytes through the T cell receptor (TCR) complex with agonists, such as an anti-CD3 monoclonal antibody, the bacterial superantigens, toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin B (SEB), and phytohemagglutinin, leads to *de novo* expression of the CD4 antigen on the cell surface which results in susceptibility of CD8 T cells to HIV infection (Flamand *et al.,* 1997). Conditioned media from

PHA or SEB stimulated CD8 T cells were not found to induce CD4 on resting CD8 cells suggesting that a soluble factor released by activated cells is not responsible for CD4 induction and that direct activation by an antigen or mitogen is required for CD4 induction. CD4 mRNA could be detected in mature CD4 T cells but there was no CD4 mRNA expression detected in the resting CD8 T lymphocytes. In addition to these findings, activation of peripheral blood mononuclear cells from HIV-infected individuals results in the appearance of T cells double-positive for CD4 and CD8, which become infected by HIV isolated directly from infected individuals.

Finally HIV-1 attachment and entry in CD8 lymphocytes and NK cells may occur via a CD4 independent mechanism and involve one of the chemokine receptors discussed earlier. CD8 lymphocytes, in common with CD4 lymphocytes, and macrophages express the CCR5 receptor (M Dittmar, personal communication).

The mechanism of HIV entry into NK cells must occur without the use of the CD4 cell receptor as they do not express CD4 nor mRNA for CD4 and infection *in vitro* is not blocked by anti-CD4 (Scottalgara *et al.,* 1993). HIV-1 may infect NK cells as they bind infected target cells in order to destroy them via a lytic mechanism. This immunological reaction requires the close apposition of the target and effector cell to avoid the release of toxic molecules into sites where they could do damage. Thus if lytic molecules are released from the NK cell to an HIV-1 infected target molecule then virus may be transmitted by cell-to-cell contact thus gaining entry into the cell without CD4.

3.16 The loss of immune function and immune cells in AIDS and HIV infection.

The decline in CD4 lymphocytes and the initial increase in total CD8 counts in most HIV infected individuals is well documented (Pantaleo *et al.*, 1993). However recent findings have shown that CD8 naive cells are depleted during the asymptomatic stage of HIV infection and that this loss can parallel that seen in CD4 lymphocytes. The increase in total CD8 counts in most HIV infected individuals is primarily due to an expansion of the memory cells. We have shown in this study that during the progression of HIV infection naive cells are preferentially lost both in the CD4 and CD8 T cell subsets. It has also been shown that in individuals with CD4 counts less than 200 per μl memory T cells were reported to account for 80% of T cells whereas this figure was only 15% in uninfected individuals. This selective decline in T cell subsets is very relevant in HIV infection and in AIDS as naive cells are required for all new T cell mediated immune responses and are memory cell precursors. Their loss, which precedes the eventual loss of memory cells, may contribute substantially to the eventual loss of the total CD4 and CD8 population (Calabro *et al.*, 1995; Rabin *et al.*, 1995; Roederer *et al.*, 1995).

Loss of naive cells has important consequences for the development of immune responses in HIV infected individuals. As naive subsets disappear, there is a progressive inability to mount responses to novel antigens, which may well result in a greater susceptibility to previously unencountered opportunistic infections. This loss would compromise the ability to deal with the constantly

mutating virus; novel strains which are immunogenetically unique, will encounter less resistance from T cell immunity than early in the progression of disease.

Recently it has been reported that among other defects, patients with AIDS display abnormalities in NK activity and that NK cells decrease in HIV-1 infection through the selective depletion of the CD16-positive, CD3-negative, CD8-positive subset (Mitchell *et al.,* 1994; Brenner *et al.,* 1997). This is the subset of NK cells reported to be infectable with HIV-1 *in vitro*. Purified DCs were reported to stimulate a strong antibody response against both the envelope and core proteins, however by the onset of AIDS antibody responses induced by DCs were low (Roberts *et al.,* 1994). DCs are also important antigen presenting cells and are involved in the stimulation of T cells. Infection, depletion and impaired function of DCs were shown to occur early in HIV infection (Macatonia *et al.,* 1990).

The immunodeficiency observed in AIDS may be attributed to the destruction of T helper cells, given its central role in all aspects of humoral and cellular acquired immune responses. However, direct infection with cells other than T helper cells contributes substantially to disease in AIDS (Watkins *et al.,* 1990). Ho *et al* (1995) examined the dynamics of CD4 T lymphocyte recovery after treatment with anti-retrovirals and found a rapid rise in the number of circulating lymphocytes. The findings suggested a high pre-treatment rate of CD4 lymphocyte cell destruction *in vivo* and a continuous process of active replacement of lost lymphocytes. The gradual decline in CD4 levels could be a failure of homoeostatic mechanisms to adequately replace cells. Therefore, although a variety of

mechanisms for cell depletion have been proposed, it is possible from the findings reported here, that the observed decline in CD4 T cells, CD8 T cells, DCs and NK cells may be a direct consequence of the cytopathic effect of HIV infection. Furthermore it is possible that this direct infection may be responsible for the impaired immune functions of each of these cells observed upon disease progression.

It is also important to note the close interactions of different cell subsets when mounting an immune response to a foreign antigen. If infection can occur by cell-to-cell contact then the immune response to HIV infection may result in a greater rate of transmission to uninfected cells. For example DCs efficiently transmit HIV to CD4 T cells and may represent explosive sites of viral infection resulting in the formation of syncytia.

Overall this study conclusively demonstrates that HIV has a broader tropism for different cell subsets within the peripheral blood in vivo than previously described. It may be that loss of these cells and their function in AIDS is a direct effect of their infection with HIV-1 and is not secondary to the loss of T helper cell function as previously postulated.

3.17 Future research

It has been reported that provirus containing lymphocytes in PBMCs are generally transcriptionally inactive and infection may be defective or virus expression may be inhibited. In order to determine whether different cell types are

productively infected with HIV-1 and if cell loss could be directly due to the cytopathic effect of viral infection, a PCR method for detection of splice HIV mRNA trancripts could be designed. If HIV mRNA could be detected in CD8 lymphocytes it would rule out thymocyte infection as a mechanism of viral entry and support infection of mature CD8 T cells. CD8 lymphocyte cultures could also be used to compare cell loss *in vitro* and determine whether more cells were lost as a consequence of HIV-1 infection.

It would also be interesting to monitor serial samples from individuals before and during anti-retroviral therapy. The frequency of infection of different cell types and cell counts could be compared as could the emergence of resistant mutants in each cell type. This would give a greater insight into cell and virus turnover rates in each cell subset.

It will be important to compare the viral load in memory and naive CD4 T cells and CD8 lymphocytes with the observed loss of these cell fractions. The emergence of anti-retroviral resistant mutants within these cell types would also be useful. These results may help to explain the preferential loss of naive cells with disease progression.

In attempt to look at the distribution of HIV in tissue, lymph node and spleen samples from *post mortem* were gently manipulated in cell culture medium to disaggregate cells. However very few viable cells were available for cell separation and PCR. In addition if HIV was detected by PCR in a purified cell fraction it would be impossible to determine whether the cells were from tissue or

the peripheral blood supply. *In situ* PCR would allow the detection of HIV-1 DNA in individual cells in tissue, which could be stained with monoclonal antibodies to specific cell markers.

The high virus load in AIDS may facilitate infection of a wide range of cells through a non-CD4 dependent mechanism. Therefore, it will be important to determine the distribution of chemokine receptors on different cell subsets. This will be easier to carry out when a panel of monoclonal antibodies to these receptors are available. It will be useful to use immunohistochemistry to determine CD4 and chemokine receptor expression on HIV infected cells.

It may be that the extensive sequence diversity of HIV amongst variants recovered from PBMCs represents a combined contribution from a variety of different population of infected cells. Therefore HIV from different cell types could be sequenced, for example in the V3 region which has been linked with cell tropism.

CHAPTER 4 : Detection of HIV-1 mRNA in CD4 lymphocytes, CD8 lymphocytes and monocytes isolated from the peripheral blood of seropositive individuals.

INTRODUCTION

4.1 HIV-1 Messenger RNAs

In HIV-1 infection the full-length, polyadenylated transcripts provide both genomes and mRNAs for the *gag* and *pol* genes. In addition single and multiple splice events give rise to over 30 alternatively processed mRNAs that are translated in the cytoplasm to produce the envelope glycoprotein and accessory proteins (reviewed in Coffin, 1996; Luciw, 1996) (figure 4.1).

Splicing occurs after transcription and is a process which not only achieves joining of the exons (figure 4.2), it also maintains them in the same order as the template DNA (reviewed in Guntaka, 1993; Levy, 1993; Coffin, 1996; Luciw, 1996). Conserved sequences have been demonstrated at the exon-intron junctures, GU at the 5' splice site, AG at 3' splice site and another short sequence called a branch sequence, located 30 bases upstream of the 3' end of the intron. It is thought that splicing enzymes recognise some common configuration of the mRNA and with the help of small nuclear ribonucleoprotein particles, or snRNPs, catalyse the cutting and splicing reactions. snRNPs are composed of RNA and protein and the RNAs of these particles have nucleotide sequences complementary to those at the exon-intron junctions or the branch sequences. Five different kinds of snRNPS are involved in splicing and may act to align the splice sites by hydrogen bonding to the sequence at the intron-exon boundaries. The RNA of a U1 snRNP base pairs with the 5' intron junction and a cut is made. This reaction creates a loop or a lariat-

Figure 4.1 : HIV-1 mRNA transcripts. ▼ represent the position of primers designed to detect HIV-1 mRNAs.

HIV-1 mRNAs

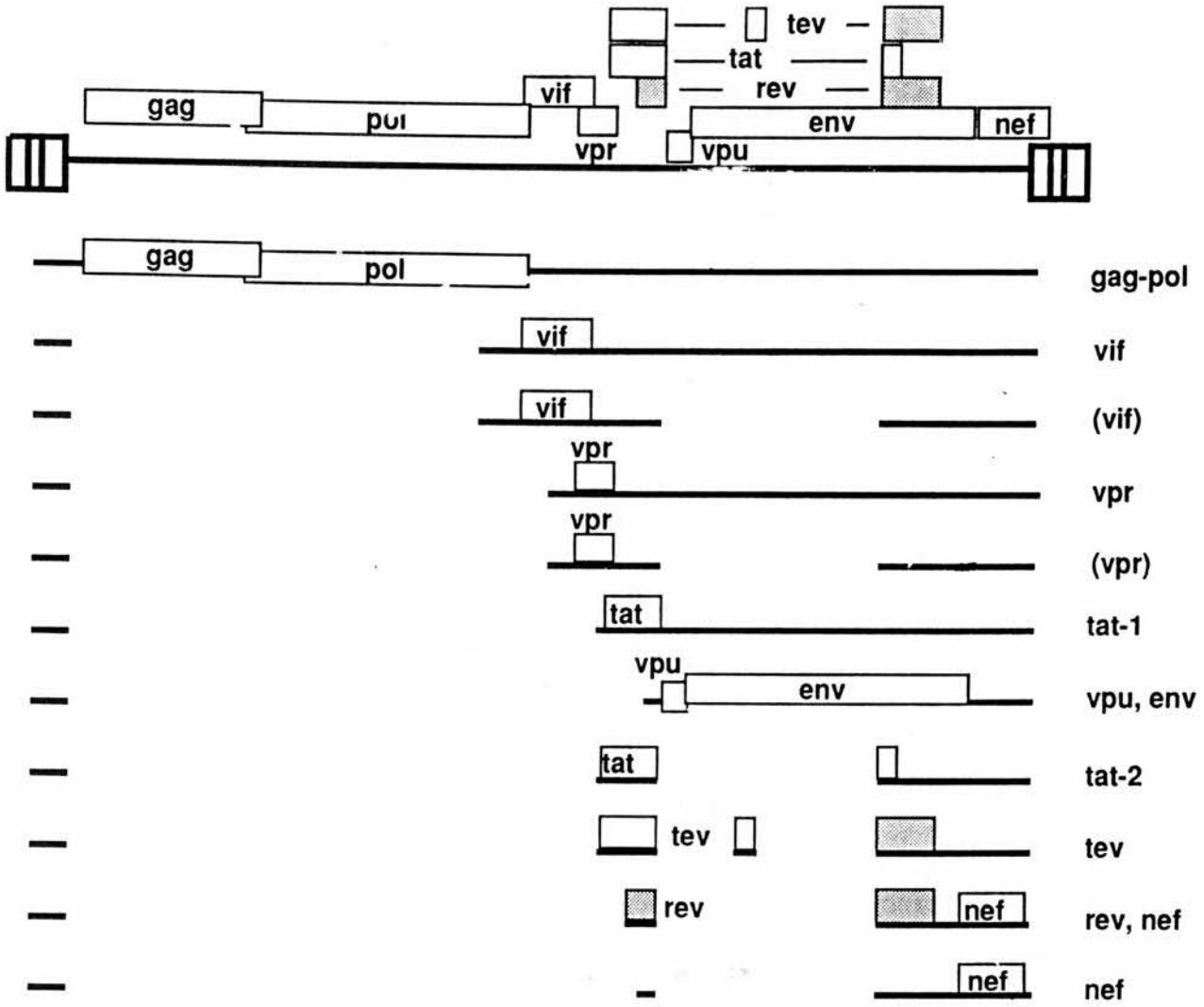
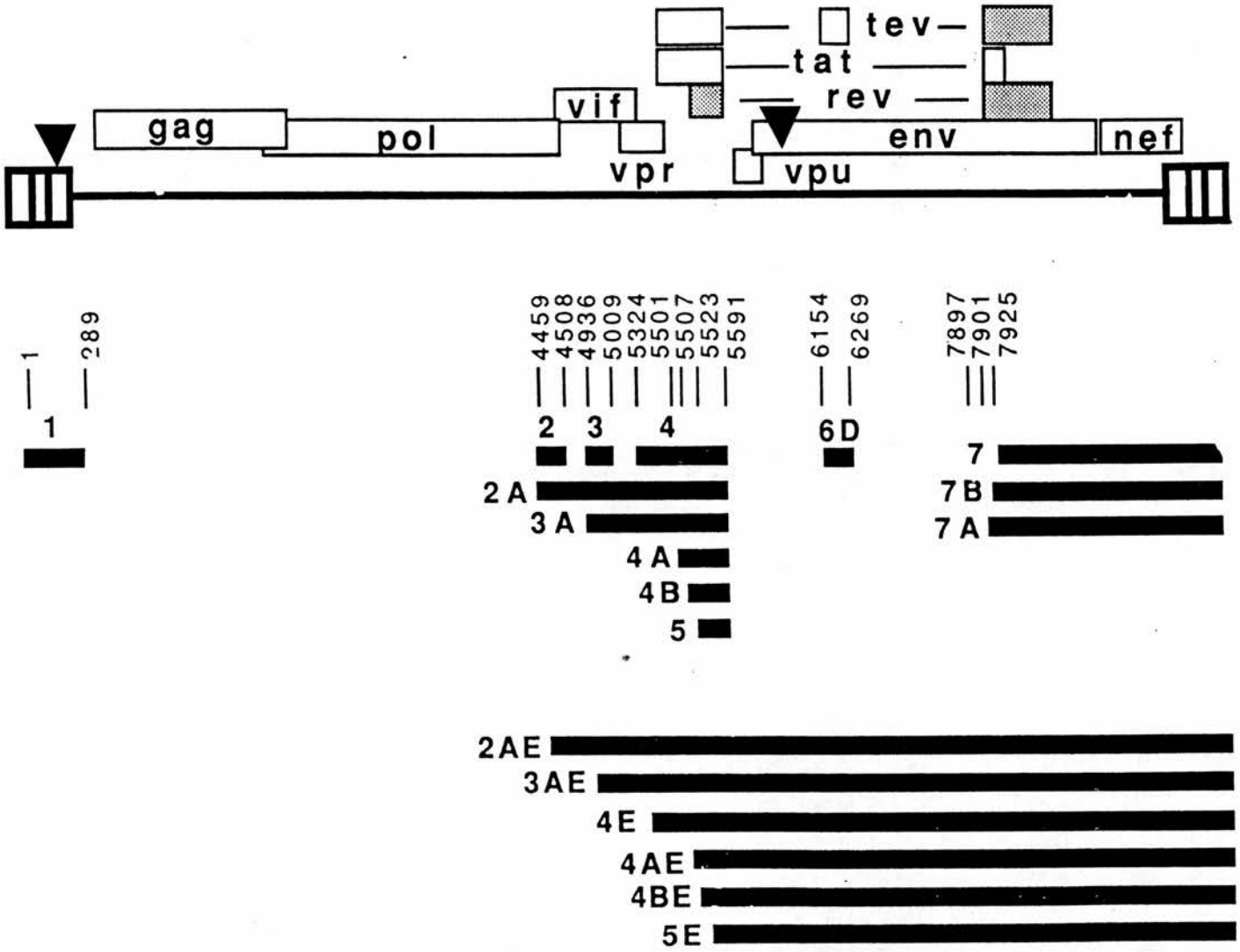

tev
tat
rev
gag
pol
vif
vpr
vpu
env
nef
gag-pol
vif
(vif)
vpr
(vpr)
tat-1
vpu, env
tat-2
tev
rev, nef
nef

Figure 4.2 : HIV-1 exons. ▼represent the position of primers designed to detect HIV-1 mRNAs. Splice donor and acceptor sites are numbered according to their positions in the HXB2 genome.


tev
tat
rev
gag
pol
vif
vpr
env
nef
vpu
1
289
4459
4508
4936
5009
5324
5501
5507
5523
5591
6154
6269
7897
7901
7925
1
2
3
4
6D
7
2A
3A
7B
7A
4A
4B
5
2AE
3AE
4E
4AE
4BE
5E

shaped intermediate with the aid of a U2 snRNP. The newly formed 3' OH group then cleaves the 3' splice site releasing the partly looped intron and at the same time joining the two exons. These final steps are thought to involve a U5 snRNP.

In all retroviruses the spliced RNAs share 5' and 3' ends with the genome, and most subgenomic RNAs share the same 5' donor, the major splice donor. However, it has been demonstrated that following mutation of the major splice donor, the kinetics of RNA and protein synthesis are slowed. It was shown that an alternative cryptic major splice donor signal, four bases down stream was activated. The efficiency of RNA splicing from this cryptic donor (AGA^ GUACGCC) may be lower than that from the major donor site (CUG^GUGAGUA) and would explain the delayed replication and infection kinetics (Purcell *et al.*, 1993).

The splicing of HIV-1 RNA is complex because of the presence of both constitutive and alternatively used 5' RNA splice donor and 3' splice acceptor motifs. The varied use of these diverse splicing signals results in the synthesis of several sets of structurally different mRNAs for protein, including the viral envelope, regulatory and accessory proteins. Generally, the most highly spliced forms of RNA, that exclude non-coding exons, are most common except in the case of *nef*, in which the inclusion of a non-coding exon is favoured. Sixteen alternative mRNAs may encode gp160 *in vitro*. However, most of these exist at very low levels and the most common *env* is the shortest possible transcript. If any of the alternative splicing mechanisms were to occur *in vivo*, two determinants could

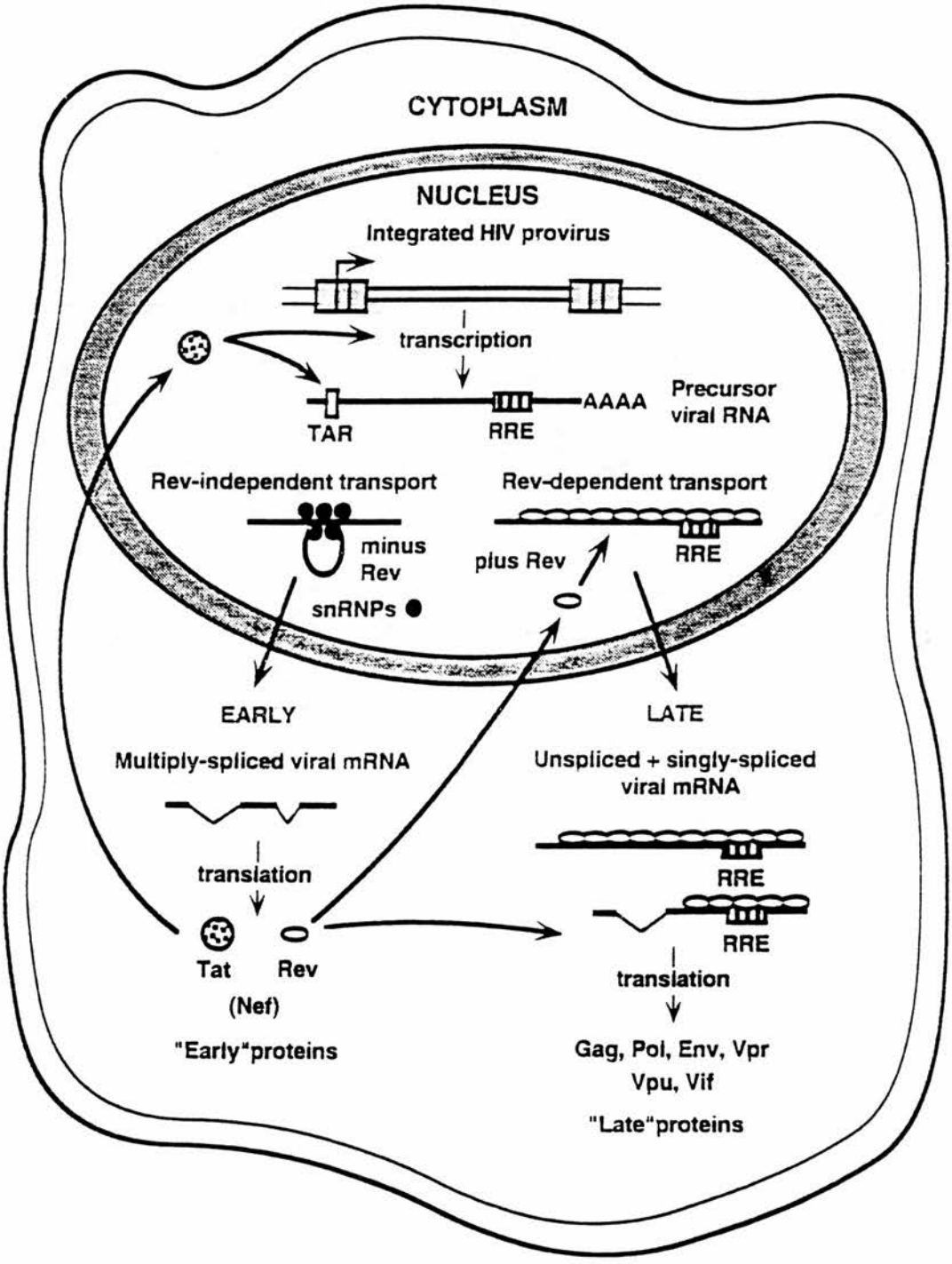
control splice acceptor usage. Firstly, the different sequence structure of the competing splice acceptor motifs in individual HIV-1 strains could alter the balance of the splice acceptor usage. It has been confirmed that HIV-1 strains with different sequences do have different splicing patterns. Or alternatively the activation status or type of cell harbouring an HIV-1 provirus could also affect the balance of splice acceptor usage.

4.2 Rev : A regulator of HIV-1 gene expression.

The ratio of spliced to unspliced viral mRNA is controlled by the regulator of viral gene expression Rev which functions through a Rev-responsive element (RRE) in full length transcripts. As Rev accumulates in an infected cell it increases the cytoplasmic levels of unspliced and partially spliced HIV-1 mRNAs, resulting in the efficient expression of the structural viral proteins (figure 4.3) (Slepushkin *et al.*, 1992).

The HIV-1 Rev protein binds viral RNA species that contain the Rev-responsive element (RRE), located in the *env* gene, thereby promoting the export, and possibly the stability and translation, of partially spliced and unspliced RNAs from the nucleus into the cytoplasm for its translation and/or packaging into progeny virions. In addition, the *rev* gene product may influence translation of viral mRNA on cytoplasmic polysomes.

Figure 4.3 : Model for Rev transactivation (From Fields Virology 1996).


CYTOPLASM
NUCLEUS
Integrated HIV provirus
transcription
AAAA
Precursor viral RNA
TAR
RRE
Rev-independent transport
Rev-dependent transport
minus Rev
plus Rev
RRE
snRNPs
EARLY
LATE
Multiply-spliced viral mRNA
Unspliced + singly-spliced viral mRNA
translation
RRE
RRE
Tat
Rev
(Nef)
translation
"Early"proteins
Gag, Pol, Env, Vpr
Vpu, Vif
"Late"proteins

4.3 Splicing Patterns and HIV-1 disease progression.

Numerous studies have demonstrated that a substantial proportion of HIV-infected individuals have ongoing viral transcription in PBMCs (Schnittman *et al.*, 1991; Arens *et al.*, 1993). In one particular study a high frequency of viral transcription was seen regardless of whether the patients were asymptomatic or had developed AIDS (Schnittman *et al.*, 1991). Individuals who demonstrated disease progression showed either a general increase in the amount of expression of all transcripts or elevated levels of unspliced transcripts in late-stage disease (Slepushkin *et al.*, 1992). In contrast a study of long-term asymptomatic individuals, undetectable or low levels of the three classes of HIV-1 transcripts (unspliced, singly spliced and multiply spliced) were detected (Michael *et al.*, 1995).

A direct association between the presence of message for a structural protein, and a more advanced immunosuppression has been demonstrated (Schnittman *et al.*, 1991). These findings are supported by a later study showing that the shift from a predominantly spliced or regulatory viral mRNA pattern to a predominantely unspliced pattern is associated with disease progression (Neumann *et al.*, 1994). It was therefore suggested that both HIV-1 cellular RNA load and splicing patterns may be used to predict disease progression. However, Saltarelli (1996) found no evidence for major changes in splicing patterns with disease progression within an individual and concluded that HIV-1 transcriptional patterns are viral strain specific rather than disease stage specific.

4.4 Development of HIV-1 mRNA RT-PCR

(i) RNA extraction

Numerous RNA extraction methods have been documented for the extraction of HIV-1 mRNA (Schnittman *et al.,* 1991; Arens *et al.,* 1993; Neumann *et al.,* 1994). In order to detect HIV-1 mRNA it was necessary initially to optimise conditions. This was achieved by analysis of cells isolated from IIIB cultures and HIV-1 negative controls. Serial dilutions of cells were extracted using various methods including proteinase K (section 2.2), RNAzol, mRNA commercial extraction kit (R & D systems), (section 2.5 iii) and a whole RNA extraction kit (Stratagene). Primers spanning the HIV-1 V3 region (section 2.3) and the constitutively expressed cellular genes β-actin mRNA (section 2.9) were used in reverse transcriptase and PCR reactions (sections 2.8 i and 2.3) in order to determine the optimal extraction method. The levels of HIV-1 RNA extracted using each method could be compared as could the levels of HIV-1 proviral DNA contamination from controls run with no RT. The whole RNA extraction kit (Stratagene) gave the most consistent levels of RNA and a low level of DNA contamination. To reduce non-specific bands from residual DNA, the extracted RNA was treated with DNAse RQ1 (section 2.7 iv).

(ii) Primer design

In order to specifically amplify HIV-1 mRNA, primers complementary to a region in the 5' LTR and the *env* region were designed (Nars, T5480, S5326, and

S5327) (section 2.8) (figures 4.1 and 4.2). In designing primers nucleotides complementary to conserved regions of at least 15 nucleotides were used with melting temperatures between 55 and 72°C (G/C = 4°C, A/T = 2°C). The melting temperature of primer pairs used in each PCR reaction were balanced as closely as possible, and palindromes and GC runs were avoided.

(iii) RT-PCR

The RNA preparation and outer-antisense primer (S5326) were warmed for 90 seconds at 65°C to disrupt any secondary structure and to allow the primer to bind to the complementary sequence on the RNA. The reaction buffer for the reverse transcriptase reaction was then added and reverse transcriptase and PCR reactions were carried out. Ten fold serial dilutions of cells from a IIIB culture were analysed as described (section 2.8) and compared with the detection of RNA by actin RT-PCR (section 2.10). Using these two PCR techniques in parallel it was possible to optimise the HIV-1 mRNA RT-PCR as the actin control reactions eliminated variations due to the efficiency of the extraction procedure. The optimal reaction conditions are given in section 2.8.

The RT-PCR technique discussed above was then compared with the Access RT-PCR system (section 2.10). The Access RT-PCR proved to be more sensitive and primary product bands less diffuse. Separate RT and PCR methods were used as it allowed limiting dilution analysis of the cDNA from the RT step, where the Access PCR does not as the RT and PCR reactions take place in one step. A control

amplification lacking RT was performed on each RNA sample to test for DNA contamination of the RNA samples and another control lacking template was also performed to monitor for contamination during processing. PCR product from spliced transcripts could be detected as distinct bands of a size differing from that of any contaminating proviral DNA or genome RNA.

The expected PCR products using the primers described, were *vif* (1431 bp), *vpr* (900 bp), *tat* exon 1 (515 bp) and *vpu-env* (317 bp) (table 4.1). Molecular weight markers (Promega) were used when analysing the PCR product on agarose gels, to confirm that the PCR products were of the predicted sizes (figure 4.4). Band A is of a length similiar to the expected *vpu-env* product, and band B is approximately 500bp in length, the product size expected from amplification of *tat* exon 1. After limiting dilution PCR the non-specific bands are no longer amplified and the most highly expressed mRNA is detected as a single band. In later amplifications a product of approximatly 900bp was detected corresponding to *vpr* mRNA.

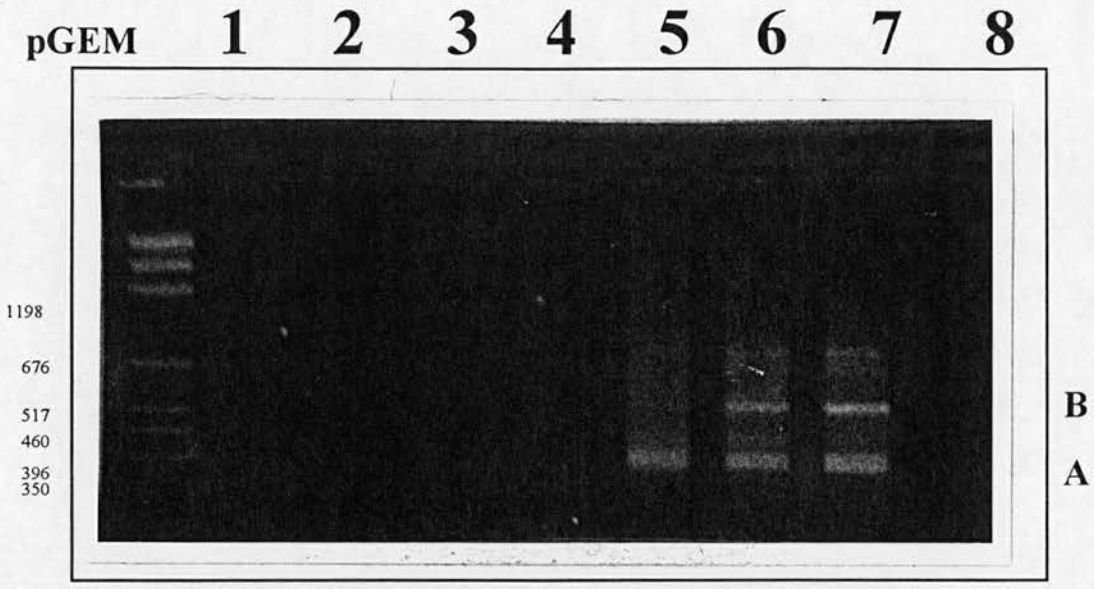
Figure 4.4 : An agarose gel illustrating HIV-1 mRNA transcripts amplified by RT PCR. In each case IIIB cell cultures were used. pGEM molecular weight markers were used to estimate the band lengths.

GEL A : Primary PCR product. Lane(1) HIV-1 negative PBMCs, (2) HIV negative Hela cells, (3) HIV negative PHA stimulated PBMCs, (4) HIV-1 negative plasma (5) IIIB stock control culture PBMCs (1 µl RNA in PCR reaction), (6) IIIB (0.1 µl RNA), (7) IIIB (0.01 µl RNA), (8) IIIB (0.001 µl RNA).

GEL B : Secondary PCR product. In lanes 9 to 12, 1 µl of primary product from lanes 5 to 8 respectively were used in secondary reactions. In lanes 13 to 16, 0.1 µl of primary product from lanes 5 to 8 respectively were used in secondary reactions.

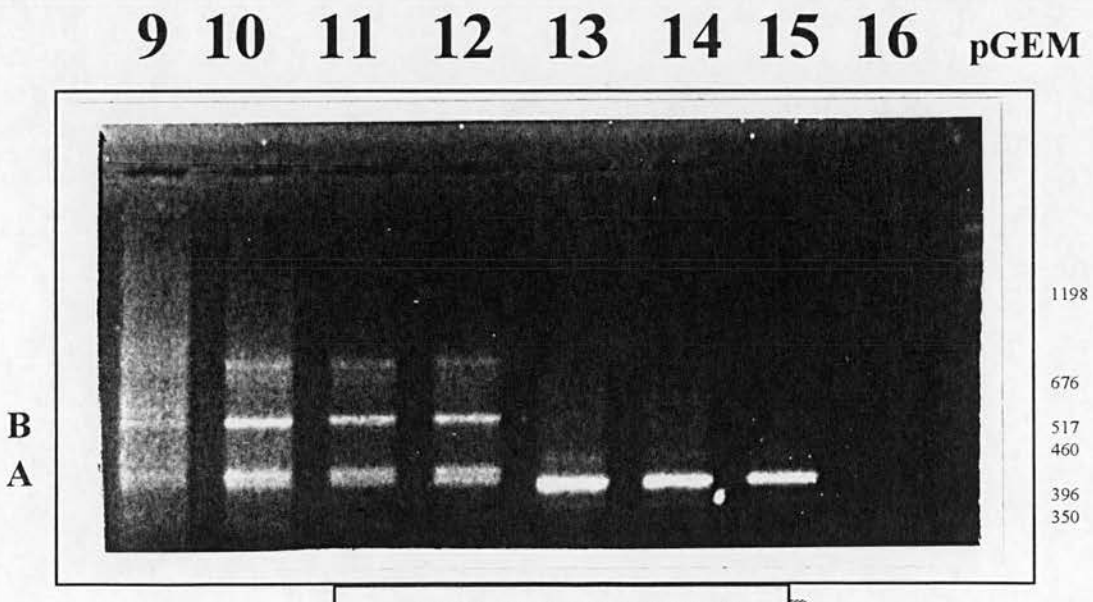
Band A is approximately of the length expected for *vpu-env* transcripts. Band B is approximately 500bp the product size expected for amplification of *tat* exon 1.

A



PRIMARY PCR

B



SECONDARY PCR

Table 4.1 : Product sizes expected from HIV-1 mRNA PCR

	Position in HXB2 Genome	Product Size (base pairs)
5' Major splice junction	743	NA[a]
Primer T5480	693	NA
Pimer S5327	4912	NA
vif 3' Splice acceptor	6293	***(vif)* 1434**
vpr 3' Splice acceptor	5388	***(vpr)* 900**
tat exon 1 3' Splice acceptor	5777	***(tat* exon 1) 515**
vpu-env 3' Splice acceptor	5976	***(vpu-env)* 317**

a NA = Not applicable

(iv) Sequence analysis

From the dilutions of the extracted IIIB controls, the PCR-end point cDNA from the RT step was diluted and serial PCR reactions carried out using a biotinylated inner primer (Primer S5327 section 2.5 i). Diluting the cDNA meant single mRNA molecules, or only one splice transcript product was amplified and these single bands were sequenced using the primer T5480 (section 2.5 ii-v). In each case the sequences obtained were of HIV-1 mRNA for *tat*, *vpu-env* (figure 4.5 A), or *vif* (figure 4.5 B) with the intron sequences spliced out. Only a small

Figure 4.5 : Sequence analysis of HIV-1 mRNA products showing changes from the HXB2 consensus. n represent any base. Figure 4.5A: LTR-Vpu, env transcripts sequenced from IIIB positive controls and CD4 lymphocytes isolated from S14. Figure 4.5: LTR-Vpr transcripts sequenced from IIIB controls and CD8 lymphocytes isolated from S14.

Figure 4.5(A): LTR-VPU,Env mRNA Sequences

Position HXB2	728	751		5976	6052
HXB2	GAGGGGCGGCGACTG	*SPLICE*	GAAGAAGCGGAGACAGCGACGAAGAGCTCATCAGAACAGTCAGACTCATCAAGCTTCTCTATCAAAGCAGTAAGTAG		
IIIB	.....	*SPLICE*	.....nn.....**T**....nnn.		
S14 CD4	.....	*SPLICE*	..**GA**.....**A**.....**CA**..**A**.....**C**.....nnn.		

Figure 4.5(B): LTR-VPR mRNA Sequences

Position HXB2	751		5388	5472
HXB2	GGCGACTG	*SPLICE*	GACTCTGCTATAAGAAAGGCCTTATTAGGACACATAGTTAGCCCTAGGTC.TCAATATCAAGCAGGACATAACAGGTAGGATCTC	
IIIB	.....	*SPLICE*	.....nnnnnnnnnnnnnnnnnn.....	
S14 CD8	...	*SPLICE*	....nnn**A**.n.....**A**...**A**..**G**.....**GTT**.....**T**.....**A**.**A**.**G**.....nn.....	

region of the *tat* exon 1 sequences could be read and these corresponded to positions 5791 to 5859 in the HXB2 genome.

4.5 Isolating RNA from cell subsets from the PBMCs of HIV-1 seropositive individuals.

CD4 lymphocytes, CD8 lymphocytes, and monocytes were isolated from eight HIV seropositive individuals and HIV-1 negative control samples. Four positive samples and one negative control were separated using the E-rosetting and magnetic bead positive selection (section 2.1 iv and v), the remaining four HIV-positive samples and a negative sample were separated using the miniMACS system (section 2.1 vi). These separated samples were immediately stored as pellets in liquid nitrogen to minimise RNA degradation and were previously used to determine the level of proviral DNA (table 3.3 and 3.4). Whole RNA was extracted using the Statagene isolation kit and RT-PCR was carried out using primers spanning the actin intron and primers complementary to the HIV V3 region of the external envelope glycoprotein. Control RT reactions were carried out in parallel to detect any contaminating DNA.

Neither the cellular β-actin gene nor V3 HIV-1 genomic RNA could be detected in the cell samples isolated using the E-rosetting technique. This cell separation procedure takes many hours and it was concluded that any RNA present may have been degraded during the separation procedure. However the miniMACS cell separation method is considerably less time consuming and therefore RNAse

activity was limited. Both HIV-1 V3 and actin mRNA were detected in these samples. Cells isolated using the miniMACS procedure were used for all investigations involving HIV-1 mRNA RT-PCR.

RESULTS

4.6 Detection of HIV-1 mRNA in CD4 and CD8 lymphocytes and in monocytes isolated from seropositive individuals

Previously stored aliquots of separated monocytes and CD4 and CD8 lymphocytes from individuals S11, S14, S21, and S22 (tables 2.2 and 3.4) with previously determined HIV-1 proviral loads were extracted using the Stratagene whole RNA isolation kit. When it was possible to isolate enough cells, nucleic acids were extracted from ten replicates of ten-fold serial dilutions of each cell subset (approximately 10^7 to 10^1 cell), DNAse treated, and RT-PCR reactions carried out as optimised for IIIB above. The aim of this study was to estimate the number of cells actively infected with HIV-1, therefore it was essential that the dilutions were carried out before the RT-step, as one cell may express many copies of HIV-1 mRNA.

In order to determine that the efficiency of the RNA extraction and RT step was equal for each sample IIIB limiting dilution controls, with a known PCR cut-off were run in parallel with each study sample. Also, HIV RNA levels were normalised to the levels of the constitutively expressed cellular genes β-actin

(section 2.9). In each case β-actin present was detectable in reactions containing 10^6 to 10 cells but not when less than 10 cells were present in the PCR reaction. The end-point of the IIIB dilutions was also constant throughout. Therefore although this RT-PCR method cannot directly quantify the number of cells expressing HIV-1 mRNA, values calculated in each cell fraction can be compared.

CD4 counts from these four individual were 85, 354, 386, and 418 per ml whole blood (table 4.2). Only one individual studied had a CD4 count less than 200 because in individuals with advanced disease too few CD4 cells could be isolated to perform both PCR for HIV provirus and mRNA. Individual S11 had a CD4 count of 85 and an AIDS defining illness. HIV-1 mRNA was detected in all individuals except S22, which had the lowest proviral load of the individuals studied, in CD4 cells [contains CD4 lymphocytes, and DCs (section 3.9)]. HIV-1 mRNA was detected at a low level in only individual S14 when 10^6 cells were present. In the isolated CD8 lymphocytes HIV-1 mRNA could be detected in individuals S11 , S14, and S22 when a minimum of 10^4, 10^4, and 10^5 cells were extracted respectively. In order to verify that CD8 lymphocytes are actively infected with HIV-1 the PCR product was sequenced as described for IIIB (section 4.4) and spliced *vpu-env* and *vpr* sequences were obtained (figure 4.5).

Table 4.2 : Limiting Dilution HIV-1 Messenger RNA PCR for Ten-fold Dilutions of CD4 lymphocytes, CD8 Lymphocytes, and Monocytes.

					Limiting Dilution mRNA PCR							
Patient	**Risk [a] Group**	**A.D.I. [b]**	**Mean CD4 Count/μl**	**Cell Type**	**10^{6} [c]**	**10^{5}**	**10^{4}**	**10^{3}**	**10^{2}**	**10**	**1**	**Provirus copies/10^{6} Cells**
WS11	Hetero	PCP	85	**CD4 T Cell**	+	+	+	-	-	-	-	1620
				CD8 T Cell	+	+	+	-	-	-	-	19
				Monocytes	-	-	-	-	-	-	-	71
WS14	Homo	None	354	**CD4 T Cell**	+	+	+	+	-	-	-	3817
				CD8 T Cell	+	+	+	-	-	-	-	65
				Monocytes	+	-	-	-	-	-	-	5
WS21	Homo	None	386	**CD4 T Cell**	+	+	+	+	-	-	-	568
				CD8 T Cell	-	-	-	-	-	-	-	435
				Monocytes	+	+	-	-	-	-	-	0
WS22	Homo	None	418	**CD4 T Cell**	-	-	-	-	-	-	-	50
				CD8 T Cell	+	+	-	-	-	-	-	25
				Monocytes	-	-	-	-	-	-	-	286

a Hetero = Hetersexual, Homo =Homosexual.
b ADI = AIDS Defining Illness.
c Number cells in PCR reaction. + , more than one replicate positive from ten replicates.

DISCUSSION

4.7 RT PCR for HIV-1 mRNA

It proved difficult to accurately quantify the number of cells actively infected with HIV-1 mRNA for a number of reasons. Firstly, it was difficult to determine the level of RNA lost during the cell separation, RNA extraction and RT reactions due to the action of RNAses. Secondly the RNA extraction and RT reaction efficiencies may vary from sample to sample, and finally splice patterns vary from individual to individual and perhaps between cell subsets within an individual. These different splice patterns further complicate investigations as longer RNA molecules are amplified less efficiently. Therefore, because the size of the amplified band influences the overall amplification, reliable quantitations of the different mRNAs can only be made by comparison to the same band of known quantity. As shorter molecules are amplified more efficiently it is not possible to compare levels of expression of different mRNAs.

In this study *vpu-env*, or *vpu-env* and *vpr* were the only splice products detected in cells isolated from seropositive individuals. The *vpu-env* band was used in the separated cells to determine the level of HIV-1 mRNA. Also the β-actin mRNA PCR and IIIB controls normalised the extraction and RT steps and allowed comparisons to be made between individuals semi-quantitatively.

Previous reports have also noted that attempts to use RT-PCR for the amplification and identification of all the HIV-1 mRNAs have not been fully satisfactory for a number of reasons, including the generation of numerous PCR artifacts and the nonlinear amplification of various mRNAs (Neumann *et al.*, 1994). In one study this group used combinations of cloned HIV-1 cDNAs of known concentration as markers for the identification of the differently expressed mRNAs and as standards to quantify HIV-1 mRNA in PBMCs (Neumann *et al.*, 1994). This may be a strategy to adopt in order to quantify mRNA in future investigations.

4.8 Detection of HIV-1 mRNA at all stages of disease progression.

In a previous study HIV-1 transcripts were detected in 94% of the 48 individuals studied (Arens *et al.*, 1993). This level of detection was maintained whether the PBMCs were isolated from asymptomatic individuals or those with AIDS (Schnittman *et al.*, 1991). The results from the four individuals studied confirm that HIV-1 replication occurs throughout HIV-1 infection irrespective of CD4 counts and disease progression.

4.9 Productive infection of CD4 and CD8 lymphocytes *in vivo*.

The permissiveness of target cells to HIV-1 infection and replication are linked to cellular proliferation, activation and differentiation. T cell activation is required for completion of HIV-1 reverse transcription and integration. HIV transcripts were detected in CD4 cells, CD8 lymphocytes and at a low level in monocytes. In earlier studies infection of monocytes was thought to be by a slow replicating virus when compared with HIV-1 replication in T cells. The findings in this study confirm the finding that viral replication is indeed at a lower level in the monocyte population than the T lymphocytes within the peripheral blood of HIV seropositive individuals.

Provirus containing lymphocytes in PBMCs and lymph nodes are generally transcriptionally inactive (Harper *et al.*, 1986; Embretson *et al.*, 1993). It is likely that cells containing HIV-1 provirus but with no detectable virus replication represent the small proportion of cells not destroyed in HIV infection, either because of infection with a defective virus or because virus expression is inhibited. HIV-1 mRNA was detected in purified CD8 lymphocytes of three of the four individuals studied. From sequence analysis it was evident that the resulting PCR products were the spliced mRNAs *vpu-env* and *vpr*. Infection of both CD4 and CD8 lymphocytes with active viral replication, substantiates the theory noted in the previous chapter that immune dysfunction and cell loss may be a direct consequence of HIV-1 infection.

A recent study suggested that infection of CD8 lymphocytes occurs in the thymus when the cells express both CD4 and CD8 (Kitchen *et al.*, 1997). Recent reports suggest that HIV-1 infection involves continual rounds of viral replication, cell destruction and cell replacement (Ho *et al.*, 1995; Wei *et al.*, 1995). The detection of active replication in CD8 lymphocytes undermines thymic infection and gives support to the possibility that HIV-1 infection occurs in the mature population of CD8 lymphocytes in peripheral blood. If immature CD4 CD8 thymocytes were infected with HIV-1 it is likely that activation of the virus would occur upon stimulation of the CD8 T cells and the resulting virus replication would lead to the destruction of the cell before it reached the peripheral circulation.

4.10 Future work.

In order to more accurately determine the relationship between HIV-1 mRNA levels in PBMCs and disease progression further samples must be analysed. Combinations of cloned HIV-1 cDNAs of known concentration should be used so quantitation of HIV-1 mRNA transcripts can be compared with these standards. HIV-1 expression should also be investigated in dendritic cells and NK cells.

Provirus-containing lymphocytes in PBMCs and lymph nodes are generally transcriptionally inactive. It will be interesting to investigate the distribution of actively and latently infected cells in PBMCs. In particular, it will be useful to differentiate memory and naive subsets in CD4 and CD8 lymphocytes as loss of CD8 lymphocytes occurs predominantely in the naive, CD45RA subset. The

distribution of actively and latently infected cells in the naive and memory subsets of CD4 and CD8 lymphocytes will allow a more accurate evaluation of the dynamics of infected cell turnover, and provide information on the mechanism of lymphocte depletion upon disease progression.

CHAPTER 5 : HIV-1 V3 sequence variation in different cell subsets isolated from infected individuals.

INTRODUCTION

5.1 HIV-1 Genomic Variation

A striking feature of HIV infection is the diversity and rapid turnover of sequence variants within an infected individual (Tersmette *et al.*, 1988; Meyerhans *et al.*, 1989; Simmonds *et al.*, 1990). The process of reverse transcription is error prone, contributing to the high genetic variability of these viruses (Coffin, 1992). Genetic variation of retroviruses is the composite of the mutation rate per replication cycle, the number of replication cycles and the selective advantage or disadvantage possessed by the virus variant. In addition, the rate of recombination in retroviruses is high and greatly contributes to genetic variation (Berkower *et al.*, 1991).

Considerable sequence diversity is observed between different HIV-1 isolates, particularly those from geographically distinct regions, where divergence has taken place over a number of years. Sequence diversity is seen within isolates from the same individual as well as between HIV strains infecting different individuals (Levy, 1993). The level of HIV sequence variation is not uniform throughout the genome, *gag* and *pol* genes are more conserved than *env*. In particular, the region of the viral genome that encodes gp120 shows an extremely rapid rate of sequence change, concentrated into five hypervariable domains (V1 to V5) interspersed among less variable regions (C1 to C4) (Levy, 1993). It has been speculated that high rates of sequence change in these regions may have adaptive value in allowing immunological escape from the initially effective antiviral immune

response (Masuda *et al.,* 1990; Wolfs *et al.,* 1991; Wolfs *et al.,* 1991; Zwart *et al.,* 1991).

5.2 Sequence variation in the V3 region of HIV-1 gp120 and *in vitro* phenotype.

V3, the third variable domain in the gp120 subunit of HIV-1 contains 35 amino acids arranged in a disulfide loop (figure 5.1). This domain plays an important role in governing several biological properties of the virus; cell tropism, cytopathicity, and fusogenicity (Luciw, 1996). The tip of the V3 loop has been designated the principal neutralising domain because virus neutralising antibodies directed against this region block HIV-1 infection of primary lymphocytes and macrophages. The GPGRAF motif (figure 5.1 amino acids 15 to 20), located at the crown of the loop, is highly conserved among HIV-1 isolates, and sequences near the cysteine residues at the bottom of the loop also show little variability.

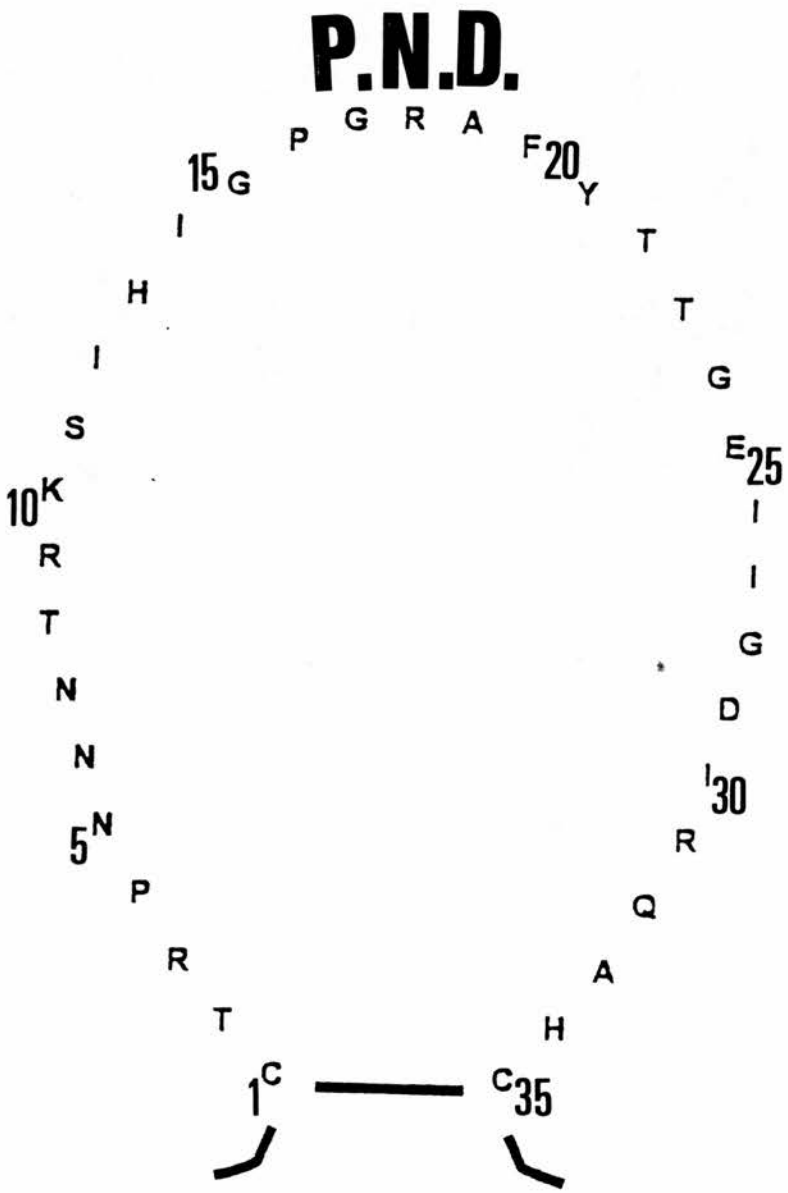
Nucleotide sequence analysis of amplified DNA from the V3 hypervariable region indicated that the initial virus population of acutely infected individuals were completely homogenous in sequence contrasting with the high degree of variability normally found in seropositive individuals and were similar to those associated with macrophage tropism in primary isolates of HIV (see below) (Zhang *et al.,* 1993). These HIV variants with a non-syncytium inducing (NSI) macrophage tropic (MT) phenotype predominate in early asymptomatic infection (Schuitemaker *et al.,* 1992). In the course of infection, HIV-1 isolates display increased replication rates,

reduced macrophage tropism and a more cytopathic syncytium inducing (SI) phenotype (Fenyo *et al.,* 1988; Cheng-Mayer *et al.,* 1989; Connor *et al.,* 1994).

Initially, SI capacity of HIV-1 isolates was determined by scoring syncytium formation upon inoculation of peripheral blood mononuclear cells (PBMCs) with virus or co-cultivation of PBMCs with virus-infected cells. Alternatively the SI capacity of HIV-1 was determined by using MT2 cells as an indicator, since MT2 cells selectively support replication of SI variants. Changes in the V3 loop have been shown to influence the phenotype of variants of HIV-1 in *in vitro* culture (Hwang *et al.,* 1991; Fouchier *et al.,* 1992; Andeweg *et al.,* 1993; Boyd *et al.,* 1993). In particular, substitutions of basic amino acids change virus isolates from NSI to SI isolates (de Jong *et al.,* 1992; Fouchier *et al.,* 1992) and may confer a reduction in the ability of the virus to replicate in macrophages (Schuitemaker *et al.,* 1991; Chesebro *et al.,* 1992).

Analysis of natural variants of HIV-1 coupled with studies on point mutations introduced into V3 in molecular clones of virus, indicates that basic amino acids in one or more positions 11, 24, 25, 29 and 32 (figure 5.1) confer a SI phenotype, whereas hydrophobic amino acids in these positions correlate with a NSI phenotype (Chesebro *et al.,* 1992; de Jong *et al.,* 1992; Fouchier *et al.,* 1992; Milich *et al.,* 1993). Fouchier *et al* and de Jong *et al* (1992) found that in NSI variants, the amino acid residue at position 11, 25 and 29 were uncharged or negatively charged. The SI phenotype was associated with a change from serine at position 11 into a naturally occurring arginine (S to R). In addition a naturally

Figure 5.1 : The HIV-1 V3 loop. Amino acids are numbered 1 to 35. The GPGRAF sequence at the tip of the loop is highly conserved between HIV-1 isolates and is designated the principal neutralising domain (PND).


P.N.D.
PRAGUE
15
20
25
30
35
10
5
1

occurring mutation at position 25, aspatate to glutamine, (D to Q) or position 29, aspartate to asparagine, (D to N) are required for full expression of the syncytium-inducing, high replicating (SI) phenotype. Further inspection of sequences of viral variants isolated from infected individuals indicates that SI, T-cell line tropic viruses also generally have a non-acidic amino acid or alanine at position 32, whereas macrophage-tropic viruses have either an acidic or neutral amino acid at this position (Fouchier *et al.,* 1992; Milich *et al.,* 1993).

The SI phenotype correlates with increased V3 sequence heterogeneity with a broad range of substitutions, insertions, and deletions at many positions between the disulfide-bridged cysteine residues (Chesebro *et al.,* 1992; Milich *et al.,* 1993). In contrast, V3 sequences from NSI isolates show few sequence differences from each other or from a consensus sequence of 133 North American isolates (LaRosa *et al.,* 1990) that comprise predominantly subtype B variants of HIV-1 (Louwagie *et al.,* 1993). The V3 domain from different HIV-1 isolates may assume two distinct conformations that determine preferential tropism for lymphocytes or macrophages (Shioda *et al.,* 1992). If the correlation between V3 sequence and viral phenotype is based on a structure-function relation, several mechanisms can be suggested. The charge and secondary structure of the V3 loop might influence binding of the virion, resulting in altered syncytium induction and infectivity or the processes subsequent to exposure of the V3 domain upon binding of gp120 to CD4 might be influenced (Sattentau *et al.,* 1991). Structure-function studies are

complicated because sequence changes out with the V3 loop may also alter the structure of V3.

5.3 Restricted sequence variability of HIV-1 V3 *in vivo*.

A highly restricted distribution of HIV was found in the body preceding the onset of AIDS, with proviral sequences apparently confined to cells of the lymphoid system (PBMCs, spleen, and lymph nodes). In contrast, those patients who died from complications associated with AIDS showed significant infection of cells in the central nervous system (CNS) and in lung and bowel tissues (Bell *et al.*, 1993b; Donaldson *et al.*, 1994b). This apparent redistribution of virus upon disease progression occurs at the same stage of disease as the apparent change from an NSI to a SI phenotype. Therefore, while isolates become cytopathic and often non-macrophage tropic *in vitro*, the redistribution of HIV *in vivo* involves organs such as brains, lungs and other tissues in which the main targets of infection are reported to be tissue macrophages, microglia (in the CNS), and other non-lymphocyte cell types.

Donaldson *et al* (1994) directly sequenced the V3 loop of virus variants in lymphoid and nonlymphoid tissues from a series of individuals who died while asymptomatic or as a consequence of AIDS, with no prior cell culture. V3 sequences from both lymphoid and non-lymphoid tissues showed highly restricted sequence variability and a low overall positive charge of the encoded amino acid sequence compared with those of standard laboratory isolates of HIV-1. The low

charge and restricted sequence variability were comparable to those observed with isolates showing a NSI and macrophage-tropic phenotype *in vitro* (Donaldson *et al.*, 1994a). All patients were either exclusively infected or predominantly infected with variants with a predicted NSI/macrophage-tropic phenotype, irrespective of the degree of disease progression.

Most of the published sequences of SI variants used for sequence comparisons were derived from isolates of HIV-1 that were passaged extensively in cell culture, therefore it is possible that whatever selection constraints restricts sequence diversity *in vivo* are absent in the conditions used for virus culture. It is also possible that the V3 loop with a large positive charge confers a growth advantage *in vitro*, leading to the selection of variants bearing such divergent sequences from a hetergenous *in vivo* population. The isolation of SI variants from patients progressing to AIDS is associated with increased virus load and therefore with a greater likelihood that such extreme variants might by chance be present in the initial PBMC culture.

Changes in the properties of isolates upon *in vitro* passaging are commonly observed. Repeat passaging enables HIV to adapt to efficient replication in different cell types, including permanently transformed T cell lines. It has also been shown that *in vitro* culture leads to rapid loss of sequence variability in the *env* gene (Kusumi *et al.*, 1992) and often to the replacement of the predominant *in vivo* variant with a minor population (Kuiken *et al.*, 1992; Kusumi *et al.*, 1992). To investigate the underlying reason for the discrepancy between the frequent isolation

of SI variants and the predicted NSI phenotype of variants *in vivo*, sequence comparisons were carried out in the V3 region from between 10-20 single molecules from PBMCs of 10 HIV infected individuals. Based upon the number of changes from the LaRosa (LaRosa *et al.,* 1990) consensus and the V3 loop charge (Donaldson *et al.,* 1994a), 3 samples could be classified as NSI only while 7 samples contained a varying proportion of more highly charged variants corresponding the SI phenotype. However, upon *in vitro* culture, all but two of the samples yielded SI isolates , in each case with evidence for the selection of the most highly charged variant in the PBMC population previously sequenced (Strappe, 1997).

5.4 HIV-1 phenotype and co-receptor usage

The chemokine receptor family members that are currently implicated as co-receptors in HIV entry include CCR5, CCR3, CCR2b and CXCR4 (section 3.2) (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996). While CD4 binds all Env proteins, CCR5 mediates viral entry into macrophages, whereas CXCR4 mediates entry into many CD4-positive transformed T cell lines (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996). However, classification of SI T cell tropic and NSI macrophage tropic HIV-1 isolates according to CXCR4 and CCR5 co-receptor usage respectively is complicated by the finding that macrophage tropic variants can also use CCR3,

while dual tropic isolates use CCR5, CCR3, CCR2b or CXCR4 (Premack *et al.*, 1996).

Recent reports have indicated that the gp120 V3 loop can influence the ability of HIV-1 variants to use different chemokine receptors (Choe *et al.*, 1996; Cocchi *et al.*, 1996). Speck *et al* (1997) demonstrated that the specific amino acids in the V3 loop of the HIV-1 envelope protein that determine cellular tropism also regulate chemokine co-receptor preference for cell entry by the virus. Comparisons of variants with Q or E at position 25 in the V3 loop regulated the ability to use CXCR4 for cell entry. The use of CCR5 was influenced by mutations at position 30.

5.5 *In vivo* V3 sequence variation in different cell subsets

Given the evidence for changes in the *in vitro* phenotype of virus isolated from PBMCs at different stages of disease progression, it is clearly important to investigate the V3 sequence of virus from different cell subsets isolated from various individuals with varying CD4 counts. In this study I carried out detailed sequence comparisons of the V3 loop and flanking regions of virus variants in CD4 and CD8 lymphocytes, as well as dendritic cells and natural killer cells isolated from five HIV-1 seropositive individuals. For solid-phase sequencing, the second PCR reaction was performed in a 100μl volume using a biotin-labelled inner antisense primer (307) and unlabelled sense primer (306). Biotinylated PCR products were sequenced with the V3 sense primer (306) as described (section 2.6).

Sequences were aligned manually and translated using the Simmonic program. Comparisons of V3 charge and amino acid sequence diversity were made between different cell subsets and individuals in this study. Rooted trees were constructed using the MEGA (Kumar *et al.,* 1993) package with the sequence HIV HXB2 as an outgroup and Jukes Cantor method to account for multiple substitutions. The bootstrap resampling method was used (100 replicates) to assess the robustness of each branch in the tree constructed.

RESULTS

5.6 Amino acid Sequence variation in the V3 loop

HIV-1 DNA extracted from different cells were diluted until only 10-30% of replicates gave a PCR product, thus ensuring that nucleotide sequences were derived from single molecules of provirus (Simmonds *et al.,* 1990b). HIV-1 V3 and flanking regions amplified from CD4 positive cells, CD8 lymphocytes, dendritic cells, natural killer cells and unseparated PBMCs isolated from individuals P6, P7, S4 (W4), S6(W6), and S24 (W5) (for patient details see tables 2.1, 2.2, 3.3 and 3.4) were sequenced and the corresponding amino acid sequences analysed (figure 5.2 to figure 5.6).

None of the nucleotide sequences in the V3 loop or flanking regions contained inactivating substitutions such as stop codons or frameshifts, supporting the previous finding (Donaldson *et al.,* 1994a) that there is little evidence for high rates of defective genomes *in vivo*. Amino acid sequences were relatively conserved

Figure 5.2 : V3 amino acid sequences and charges of HIV-1 DNA amplified from various cell subsets isolated from individual W5.

a Changes from the V3 loop LaRosa consensus sequence and flanking region HIV-MN consensus are noted.

b V3 loop charges were calculated for each virus isolate (K/S = +1, D/E = -1).

HIV-1 Consensus'		FTDNAKTIIVHRNESVQIN	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	NISRAKWNDTLRQIVSKLKEQFGKNS	CHARGE*
CD4 T Cells	1	..........L....E..	.........R..........A..D.......Y.	...K....K..E..G...R.K..D.I	+3
	2	.........QL..T.E..	.........R..........A..D.......Y.	...K....K..E.....R.K..D.I	+3
	3	..........L....E..	.........R..........A..D.......Y.	...K....K..E..S.R.S.K..D.I	+3
	4	..........L....E..	....................A..D.......Y.	...K....K..E..S...R.K..D.I	+3
	5	..........L....E..	.........T..........AA.D.......Y.		+2
	6		..........V.........A..D.......Y.	...K....K..E.V.M..R.K..N	+3
	7		....................EA.D.......Y.	...K....K..E.V.M..R.K..N	+2
	8	..........L....E..	.........R.V.......A..D.......Y.	...K....K..E..G...R.K..D.I	+3
	9	..........L....E..	..............................Y.	...KT...K..E..G...R.K..D.I	+3
	A	..........L....E..	..........V.........A..D.......Y.	...K....K..E..G...R.K..D.I	+3
	B	..........Q....E..	........GR..........A..D.......Y.	...K....K..K...I....K.	+2
	C	..........L...IE..	..........V.........A..D.......Y.	...K....K..E.....R....	+3
	D	..........L....E..	.........R..........A..D.......Y.	...K....K..E....R.R.K..D.I	+3
CD8 T Cells	1	..........L....E..	.........R..........A..D.........	...KT...K..E.V....R.K..D.I	+3
	2	..........L....E..	.........R.V.......A..D.....T.Y.	...KT...K..E.V....R.K..N.I	+3
	3	..........L....E..	.........R..........A..D.......Y.	...K....K..E.V....R.K..D.I	+3
	6	..	.........R.V.......A..D.........	...KT...K..E.V....R.K..N.I	+3
NK Cells	1		....................EA.D.......Y.	...K....K..E.V.V..R.K..N	+2
	2	..........L....E..	.........R..........A..D...N....Y.	...K....K..E.V.V..R.K..D.I	+4
	5	..........L....E..	.........R..........A..D.......Y.	...K....K..E.V.V..R....D.	+3
	6	..........L....E..	.........R..........A..D.......Y	...K....K..E.V.V..R.K..D.I	+3
PBMCS	1	..........L....E..	.........R..........A..D.......Y.	...K....K..E.....R....N	+3
	2	..........L....E..	.........R..........A..D.......Y.	...K....K..E.....R.K..D.I	+3
	3	..........L....E..	.........R..........A..D.L.....Y.	........K..E..N	+3
	4	..........L....E..	....................A..D...N....Y.	........K..E..DR.	+4
	5	..........L....E..	.........R..........A..D....M...Y.	........K..E..D	+3
	6	..........L...IE..	....................AA.D..........	.	+3
	7	..........L....E..	....................AA.D.......Y.	...	+3

Figure 5.3 : V3 amino acid sequences and charges of HIV-1 DNA amplified from various cell subsets isolated from individual W6.

a Changes from the V3 loop LaRosa consensus sequence and flanking region HIV-MN consensus are noted.

b V3 loop charges were calculated for each virus isolate (K/S = +1, D/E = -1).

HIV-1 Consensus*		FTDNAKTIIVHRNESVQIN	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	NISRAKWNDTLRQIVSKLKEQFGKNS	CHARGE*
CD4 T Cells	1	N..EN...QL....E..	..G.....R.....	TL..T...N..K..AI..R...GKN	+3
	2	...ES...QL..T.E..	.....A...V.....	T...T...S..K...I..R...KNKT	+3
	3	.....ES...QL..T.E..	.....A...V.....	T...T...N..EI..I.FR...KNKT	+4
	4		.....K.....A...V.....R.F.	TLN.T...KS.K..AI..R...KNKT	+3
	5	......I...QL....E..	.....	....T...N..K..AV..R...KNKT	+2
	6	......I...QL....E..	.....D.....	TLN.T...NA....AIE.R...RDKT	+3
	7		.....G..D.....	.L.ET..KN..KL.AI..E...GN	+3
CD8 T Cells	2	..N...N...QL..T.E..	.....AA.D.....	.L.ET..KN..KL.AI..R...KD.	+3
	3	..N..DI...QL....E..	.....P.....A..D.....	.L.ET..KN..KL.AI..R...KD.I	+3
	4	..N...N...QL....E..	.....A.....	...KT..RN..KL.AI..G...KNKT	+3
	5	..N..EN...QL..T.E..	.....A.....	...KT..KN..KL.AI..R...RNKT	+3
	6	..N...N...QL....E..	.....A.....	...KT..RN..KL.AI..R...KN.K	+3
	7		.....P.....A..D.....	.L.ET.MEN..KL.AI..R...KNKT	+3
	8	..N...N...QL....E..	.....A.....	...KT..RN..KL.AI..R...KN.K	+3
	9		.....P.....A.....	.L.KT..RN..KL.AI..R...KNKT	+3
	A	..N..EN...QL..T.E..	.....P.....A..D.....	.E.ET..KN..KL.AI..R...KD.	+3
	B	..N...I...QL..T.E..	.....P.....A..D.....	.L.KT..KN..K..AI..R...KD.	+3
PBMCS	1	..N..EN...QL..T.E..	.....A.....	T.....RN..KL.AI..R...KNKT	+3
	2	..N..EN...QL..T.E..	.....A.....	.L.ET..KN..EL.AI..R...RNKT	+3
	3	.N...QL..T.E..	.....A..D.....	.L..T..KN..EL.....R.K..	+3

Figure 5.4 : V3 amino acid sequences and charges of HIV-1 DNA amplified from various cell subsets isolated from individual W4.

a Changes from the V3 loop LaRosa consensus sequence and flanking region HIV-MN consensus are noted.

b V3 loop charges were calculated for each virus isolate (K/S = +1, D/E = -1).

HIV-1 Consensus*		FTDNAKTIIVHRNESVQIN	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	NISRAKWNDTLRQIVSKLKEQFGKNS	CHARGE*
CD4 T Cells	1	L....E..	........M.G.........A....V........	.V..E...K..E...R..R..YGNKT	+2
	2	L....E..	........M.G.........A....V........	.V..E...K..E...R..R..YGNKT	+2
	3	L....E..	........M.G.........A....V........	.V..E...K..E...R..R..YGNKT	+2
	4	..T.E..	........M.G.........A.EQ.V......Y.	.V..ER..K..E.V.K..R..YGNKT	+2
	5	L....E..	........M.G.........A....V........	.V..E...K..E...R..R..YGNKT	+2
	6	....E..	........M.G.........A.EQVV......Y.	.V..E...K..E.V.R..RK.YGNKT	+2
	7	..T.E..	........M.G.......V..A.EK.V......Y.	.V..ER..K..E.V.K..RK.YGNKT	+3
	8		........M.G.........AAEQVV......Y.	....E...K..E.V.K..RK.YGNKT	+2
	9		........M.G.........A.EK.V......Y.	.V..ER..K..E.V.K..RK.YGNKT	+3
CD8 T Cells	1		........M.G.........A.EK.V......Y.	.V..ER..K..E.V.K..RK.YGNKT	+3
	2		........M.G.........A.E..V......Y.	.VN.ER..K..E.V.K..RK.YGNKT	+1
	3		........M.G.........A....V......Y.	.V..ER..K..E.V.K..RK.YGNKT	+2
	4		........M.G.........A.EK.V......Y.	.V..ER..K..E.V.K..RK.YGNKT	+3
	5		........M.G.........A....V........	....ER..K..E.V.K..R..YGNKT	+2
	6		........M..............Q.V........	....E...K..E.V.K..RK.YGNKT	+3
	7		........M.G.........A.E.VV........	.V..ER..K..E...K..RK.YGNKT	+1
	8		........M.G.........A.EK.V......Y.	.V..ER..K..E.V.K..RK.YGNKT	+3
	9		........M.G.........A..Q.V........	.V..ER..K..E...E..R..YGNKT	+3
	A	..N.......QL..T.E..	........M.G.........A....V........	....E...K..E.V.K..RK.YGI	+2
PBMCS	1	L..T.E..	........M.G..............K.......Y.	....E...K..E.V.K..RK.YGI	+3
	2	L..T.E..	........M.G.........A..Q.V......Y.	....E...K..E.V.K..RK.YGN	+3
	3	L..T.E..	........M.G.........A..Q.V......Y.	.V..E...K..E.V.K..RK.YGN	+2
	4	L..T.E..	........M.G.........A....V........	.V..ER..K..E.V.K..RK.YGN	+3
	6	L....E..	........M.G.........A..QVV........	.V..E...K..E...R..R..YGNKT	+3
	7		........M.G.........A..Q..........	.	+3

Figure 5.5 : V3 amino acid sequences and charges of HIV-1 DNA amplified from various cell subsets isolated from individual P6.

a Changes from the V3 loop LaRosa consensus sequence and flanking region HIV-MN consensus are noted.

b V3 loop charges were calculated for each virus isolate (K/S = +1, D/E = -1).

HIV-1 Consensus*		FTDNAKTIIVHRNESVQIN	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	NISRAKWNDTLRQIVSKLKEQFGKNS	CHARGE*
CD4 T Cells	1	..QL....E..	........S.G......ST..A..RV..N....Y.	S.NGSE.....K..AI..R...IN..	+4
	2	.QL....E..	........S.G.............RV..N....Y.	SL.GSE.....K..AI..R...VN..	+5
	3	...QL....E..	........S.........R..A..R...N....Y.	SL.GS......K..AI..R..LVNI.	+6
	4	...QL....E..	........S....L...S...A..R...N....Y.	ILNGT......KK..I..R..LVDI.	+4
	5	...QL.KP.E..	........S............A..R........Y.	...GSE.....K..AI..RG.LIDTT	+4
CD8 T Cells	1	...QL....E..	........S...YL...S...A..R...N....Y.	SLNGT......KK..I..R...IH..	+4
	2	...QL....E..	........S.G.............R...N....Y.	ILNGS......K..AI..R..LEN	+5
	3	...QL..P.E..	........G.G..........A..GV.......Y.	SV.GSE..S..K..AI..R...ID.K	+3
	4	...QL....E..	........G............A..G........Y.	S.NGT...S..K..AI..R..LGN	+3
	5	...QL....E..	........G............A..RV..N....Y.	...GSE..S..K..AI..R..LGN	+5
Dendritic Cell	1	..QL....E..	........S.G..L...S...A..RV..N....Y.	S..GSE.....K..AT.VR...IN..	+4
Plasma	1	..QL.KP.E..	........S.G..M..........RV..N....Y.	...GSE.....K..AI..R...IN..	+5
	2	..QL...IE..	........S....L...S...A..R...N....Y.	SLNGT......KK..I..R...RD..	+4
	3	..QL.KP.E..	........S.G..H...S...A..RV..N....Y.	S..GSE.....K..AI..R...IN..	+4
	4	..QL...IE..	........S....L...S...A..RV..N....Y.	S..GSE.....K..AI..R...IN..	+4

Figure 5.6 : V3 amino acid sequences and charges of HIV-1 DNA amplified from various cell subsets isolated from individual P7.

a Changes from the V3 loop LaRosa consensus sequence and flanking region HIV-MN consensus are noted.

b V3 loop charges were calculated for each virus isolate (K/S = +1, D/E = -1).

HIV-1 Consensus*		FTDNAKTIIVHRNESVQIN	CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC	NISRAKWNDTLRQIVSKLKEQFGKNS	CHARGE*
CD4 T Cells	1	...QL....A..	.....R..N.....N.....	...GTE..N..Q...I..R...GN.K	+4
	2	I...QL....E..	.....R..N.....Q...N.....	.....A..N..HK..I..R...GI.K	+5
	3	...QL....A..	.....R.VN.....	.L...R..N..Q...R..R...GN.K	+3
	4	L....E..	.....R..N.....N.K..R.	.....R..N..HK..I..R...GN.K	+4
	5	.I...QL....A..	.....R..N.....N.....	.F...R..N..HK..I..R...GN.K	+5
	6	.I...QL....A..	.....RR.N.....D...N.....	.....R..N..HK..I..R...GN.K	+5
	7	.I...QL....P..	.....RR.N.....N.....	.....R..N..HK..I..R...GN.K	+5
	8	.I...QL....P..	.....RR.N.....N.....	.....R..N..HK..I..R...GN.K	+5
	9	...QL....A..	.....R..N.....	.....P..N..H...I..R...GN.K	+3
	A	...QL....A..	.....R..N.....N.....	.....R..N..HK..I..R...GN.K	+4
	B	...QL....A..	.....RR.N.....N.....	....TE..N..Q...I..RK..GN.K	+5
CD8 T Cells	1	...QL....A..	.....R..S.....N.....	....GA..N..Q...K..R...GN.K	+4
	3	.I...QL....P..	.....RR.N.....N.....	....ER..N..HK..I..R...GN.K	+5
	4	.I...QL....A..	.....RR.N.....N.....	.F...R..N.FTK..I..R...GN.K	+5
	5	.I...QL....A..	.....RR.N.....N.....	..	+5
Dendritic Cells	1	...QL....E..	.....RR.S.....A.....	.L..TA..NA.H...M..R...GN.K	+4
	2	...QL....A..	.....R..N.....D.....N.....	.F.GEE..N..K...MR.R...GN.K	+3
	3	...QL....E..	.....R..S.....N.....	....TA..N..Q...K..R...GN.K	+3
	4	...QL....A..	.....R..S.....N.....	....GR..N..Q...K..R...GN.K	+4
Monocytes	1	...QL....A..	.....R..N.....N.....	....TA..N..Q...I..R...GN.K	+4
	2	...QL....E..	.....R..S.....N.....	....TA..N..Q...I..R...GN.K	+4

in both the V3 region and flanking regions within an individual and in different cell subsets isolated from the same individual.

Amino acid sequences from individual W5 (S24) (figure 5.2) revealed changes in the 3' flanking region which were exclusive to CD4 lymphocytes (valine to glycine or serine) and natural killer cells (serine to valine). In S6 (figure 5.3) a histidine to proline substitution occurs only in the V3 loop of virus isolated from CD8 lymphocytes. A isoleucine to valine substitution at position 26 of the V3 loop was only detected in CD4 lymphocytes from individual W6 (S6). However this study provides no evidence for the existence of a shared determinant in the V3 loop that governs the distribution of HIV-1 in different cell types.

Similar diversity of HIV-1 V3 sequences was observed irrespective of the disease progression in the individuals studied. Indeed, P6 showed a relatively high level of amino acid variation from the consensus sequence when compared to the other individuals studied and P6 had the highest mean CD4 count. One of the most striking features of this study is the low level of V3 amino acid diversity within each individual. A much higher level of amino acid divergence from the consensus is observed in the V3 flanking regions when compared with the V3 "hypervariable" domain.

5.7 Prediction of in vitro phenotype from V3 loop sequences.

To investigate the relationship between *in vitro* phenotype and V3 sequence, the overall V3 charge and the degree of sequence divergence from the subtype B consensus were calculated in a previous study for a series of isolates with known biological properties. This studied showed that NSI macrophage-tropic isolates consistently showed a lower charge and/or greater similarity to the subtype B consensus than did SI and non-macrophage-tropic variants. When the number of differences from the subtype B consensus is plotted against the V3 loop charge a diagonal line separates the SI and NSI populations (figure 5.7) (Donaldson *et al.*, 1994a). This method of analysis was used to compare the *in vitro* phenotypes for individuals with differing degrees of disease progression in CD4 and CD8 lymphocytes. Clearly the vast majority of sequences analysed segregate on the left hand side of the dividing line and therefore have a predicted NSI macrophage tropic phenotype (figure 5.8). This proves true for both CD4 and CD8 lymphocytes. The 7 isolates segregated into the predicted SI section of the graph were all derived from cells isolated from P6, who interestingly, was the individual with the highest CD4 count. A plot of CD4 and CD8 lymphocyte V3 loop charges illustrates that there is no charge variation between these two cell types (figure 5.9).

Figure 5.7 : Comparisons of V3 loop sequence on the basis of predicted overall charge and number of changes fronm a sutype B consensus of sequences with a known phenotype. (Taken from Donalson *et al* 1994a).

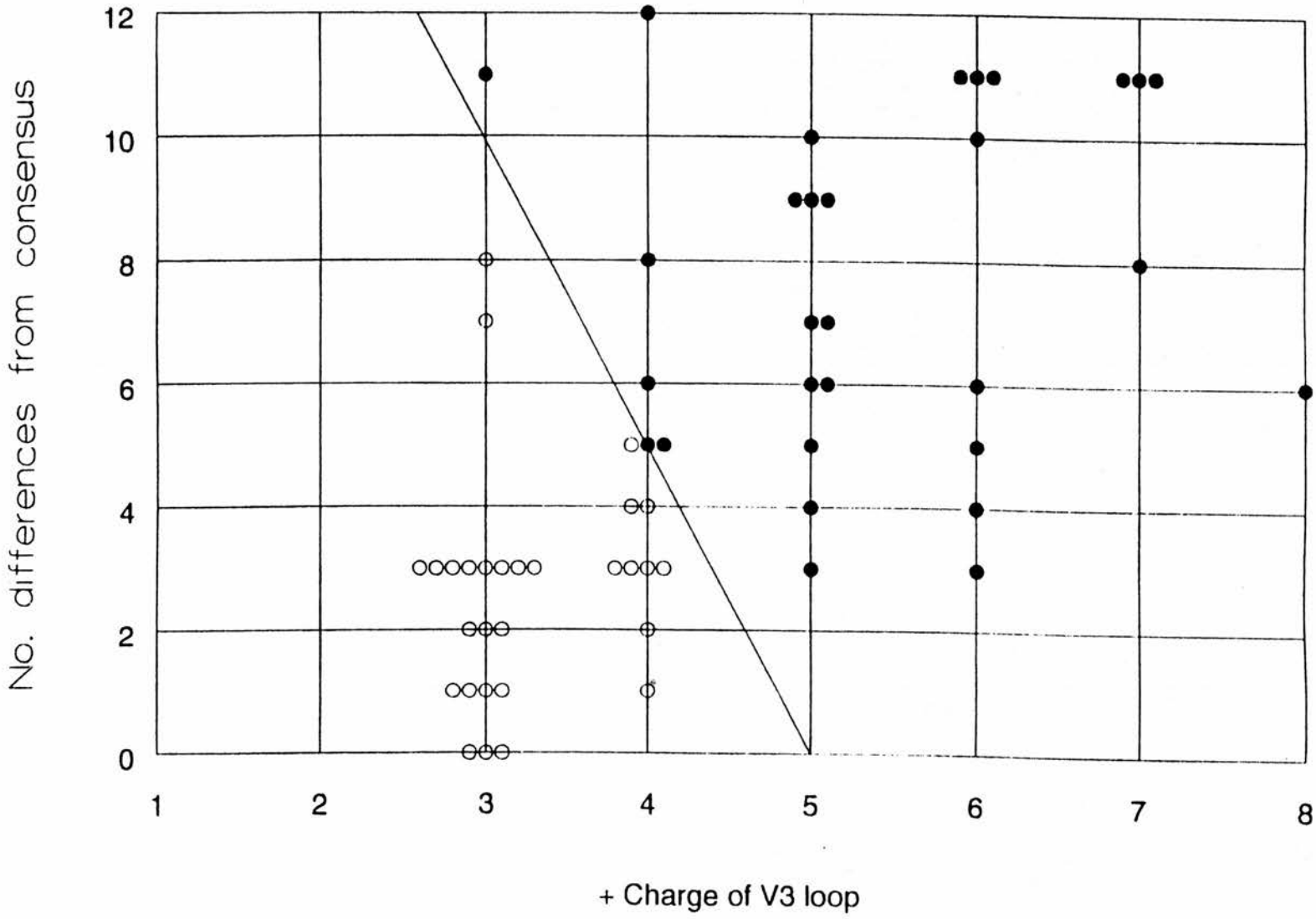

No. differences from consensus
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
+ Charge of V3 loop

Figure 5.8 : Comparisons of V3 loop sequences from CD4 and CD8 lymphocytes on the basis of predicted overall charge and number of changes from a subtype B consensus.

Filled symbols represent CD4 lymphocytes, open circles represent CD8 lymphocytes.

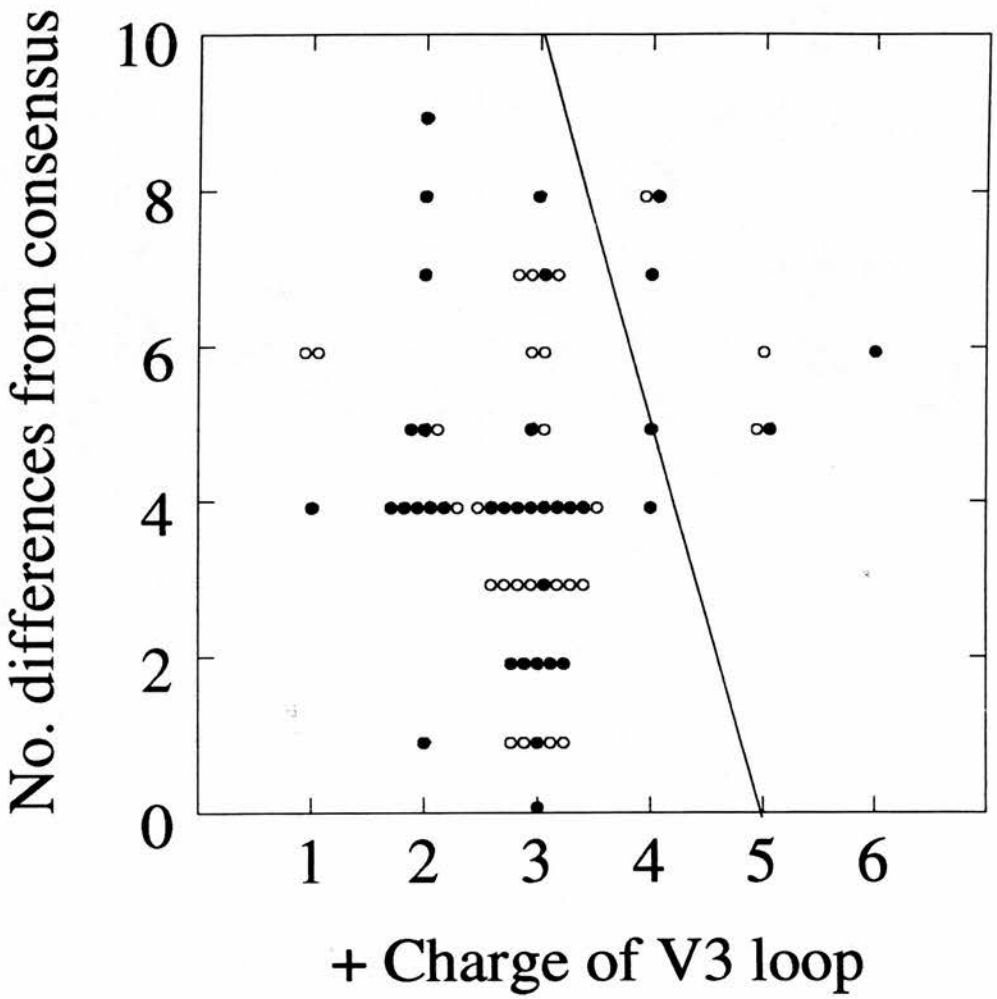
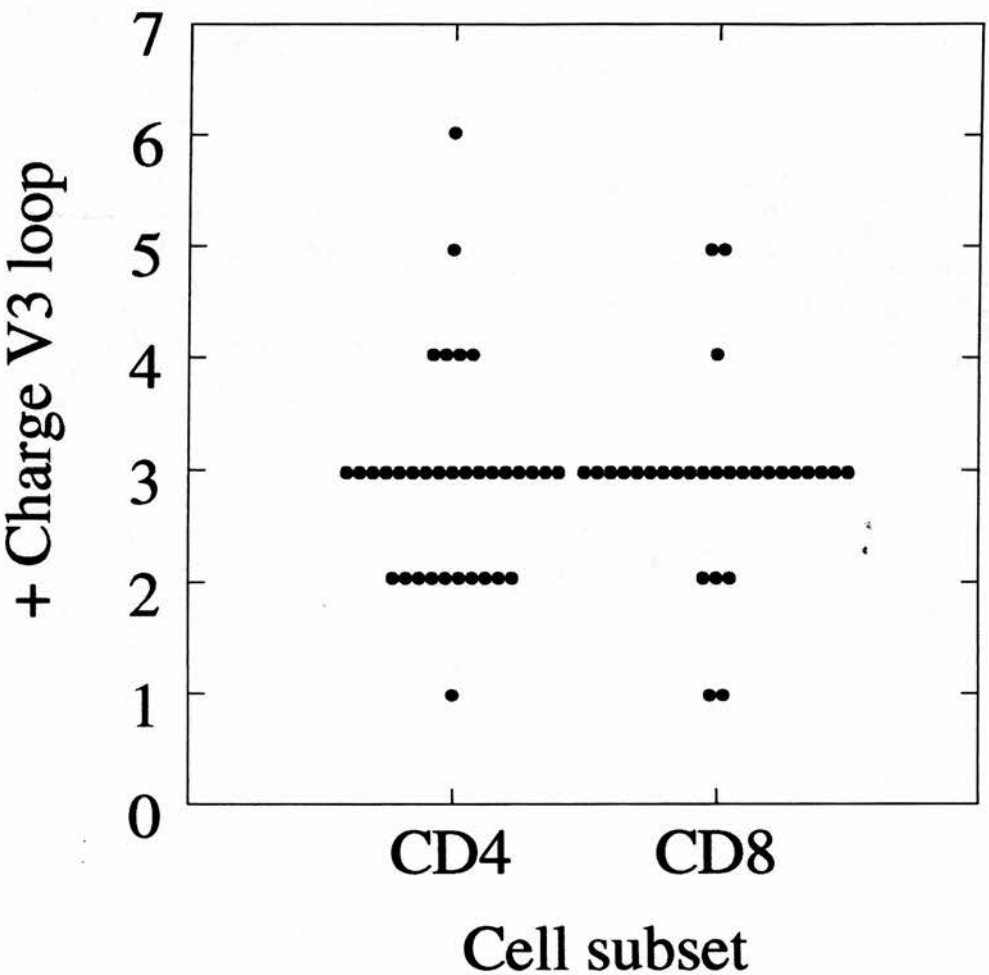

No. differences from consensus
10
8
6
4
2
0
1
2
3
4
5
6
+ Charge of V3 loop

Figure 5.9 : Comparisons of V3 loop amino acid charge in CD4 and CD8 lymphocytes.

Filled symbols represent CD4 lymphocytes, open circles represent CD8 lymphocytes.

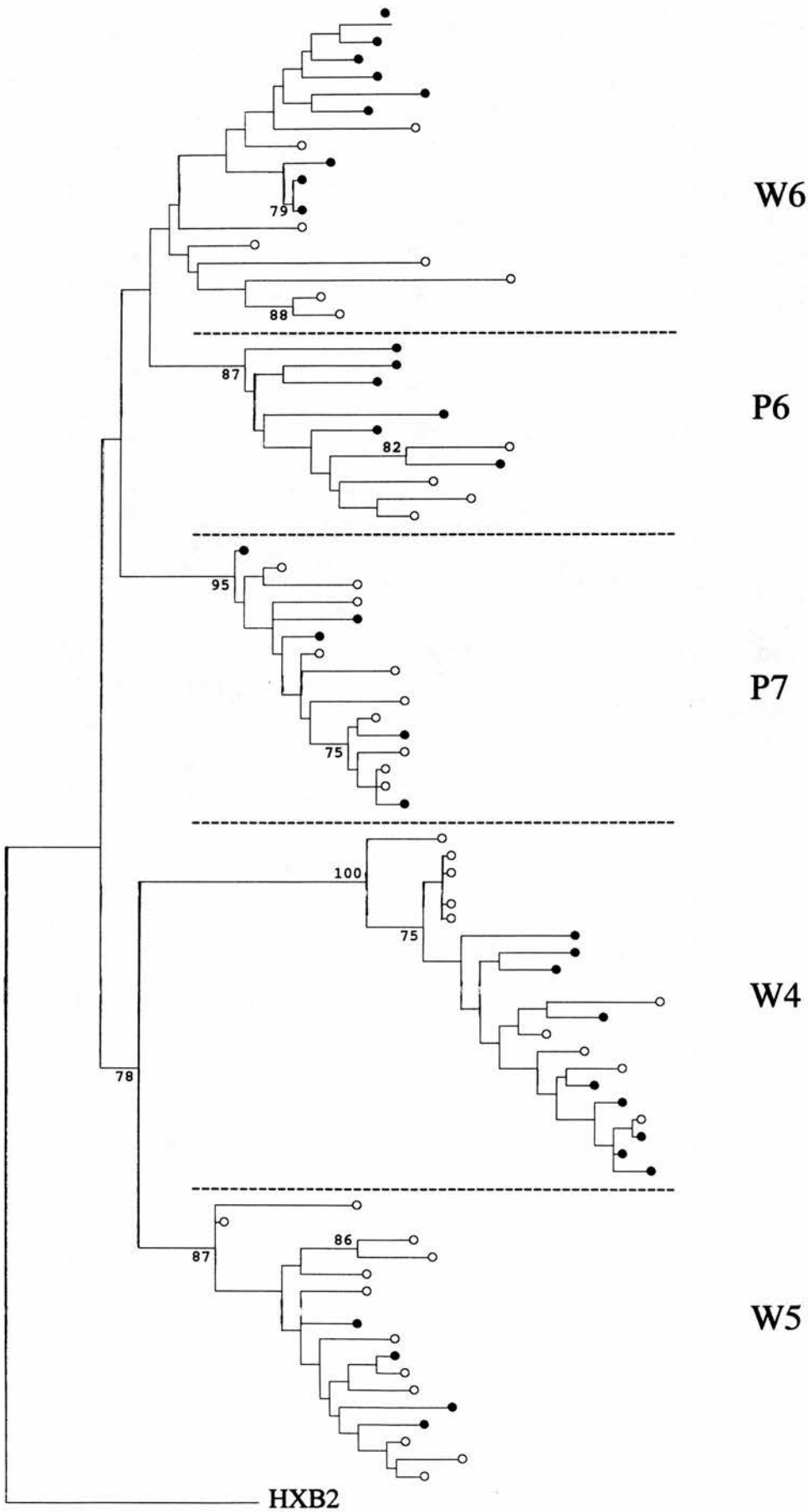

+ Charge V3 loop
0
1
2
3
4
5
6
7
CD4
CD8
Cell subset

5.8 Phylogenetic analysis of V3 nucleotide sequences from CD4 and CD8 lymphocytes.

Nucleotide sequences of CD4 and CD8 variants from five study subjects were analysed phylogenetically. The data set included the sequence of a molecular clone HXB2 to serve as an outgroup. The phylogenetic tree was constructed using a distance based method and neighbour-joining as implemented in the MEGA package (Kumar *et al.,* 1993) (figure 5.10). Pairwise distances were calculated using the Jukes Cantor method to account for multiple substitutions. Sequences from each five individuals grouped separately. To assess the robustness of the observed groupings bootstrap re-sampling was carried out using 100 iterations of permuted data. Lineages containing variants from P6, P7, W4 and W5 showed high bootstrap values indicating that variants within an individual were consistently more similar to one another than they were between individuals. There was however little bootstrap support for the existence of clearly distinct lineages within an individual although this may have been caused by the shortness of the sequences analysed (less than 300bp). Consistent with the inferred V3 amino acid sequences there was no clear evidence for separate grouping of variants from CD4 and CD8 lymphocytes although sequences from P6, W6, and to a certain extent W4 showed some evidence for distinct variants. For example sequences from CD8 lymphocytes of W6 grouped into a single claid that was distinct from all but two variants isolated from CD8 lymphocytes. Similarly sequences from the CD4 lymphocytes of P6 were monophyletic although this claid also contained one CD8 lymphocyte.

Figure 5.10 : Phylogenetic analysis of sequences in the V3 region of CD4 and CD8 lymphocytes isolated from HIV-1 seropositive individuals.

Filled circles represent virus isolates from CD8 lymphocytes. Open circles represent sequences from CD4 positive cells.



Scale: each – is approximately equal to the distance of 0.0025

DISCUSSION

5.9 Restricted sequence variation in the V3 region

In a previous study investigating HIV-1 V3 sequences from lymphoid and non-lymphoid tissues it was observed that there was limited sequence variation in the V3 loop of HIV-1 sequences amplified without prior cell culture (Donaldson *et al.*, 1994a). This study has also shown restricted V3 variation in CD4 and CD8 lymphocytes isolated from the peripheral blood of HIV-1 seropositive individuals. This restriction was evident irrespective of the degree of disease progression and cell type. It has been demonstrated that the primers used to amplify the V3 loop do not preferentially amplify NSI variants (Donaldson *et al.*, 1994a). Variants with a predicted NSI phenotype have frequently been detected in previous studies analysis viral sequences *in vivo* (Simmonds *et al.*, 1990a; Holmes *et al.*, 1992a; Kuiken *et al.*, 1992a; Milich *et al.*, 1993a).

5.10 HIV tropism *in vivo*

This study does not indicate that there is a specific V3 loop sequence which determines HIV-1 tropism for CD4 or CD8 lymphocytes *in vivo*. No single amino acid change or changes were found in the V3 loop of a particular cell type in each individual. However it is possible that V3 conformation is important in determining cellular tropism and changes out with the V3 loop may affect the conformation.

5.11 Phylogenetic analysis of the V3 region from CD4 and CD8 lymphocytes.

The V3 region is not a particularly useful region for phylogenetic analysis as this region is under both immunological and functional pressures which restrict sequence variation. The V3 loop and flanking regions analysed were also too short (approximately 300 bp) and very few robust groupings (bootstrap value >75) were detected by re-sampling. However, there was some evidence for divergent V3 nucleotide sequences in CD4 and CD8 lymphocytes from phylogenetic analysis.

5.12 Future investigations

It is difficult to know whether the different sequences represent genuine diversity of HIV within that cell type or whether each cell type purified consists of a heterogenous population of cells at different stages of activation. For example naive and memory lymphocytes may bear different HIV-1 variants. It would be useful to isolated memory and naive CD4 and CD8 lymphocytes and analyse the V3 sequences derived from each. Future samples should be of a larger volume so a greater number of cells and therefore more virus variants can be isolated for sequencing and phylogenetic analysis.

CHAPTER 6 : SUMMARY AND PROSPECTS

SUMMARY AND PROSPECTS

6.1 Summary

Infection with HIV-1 is thought to be restricted to cell types expressing the CD4 receptor. Cell types previously shown to be infected when isolated from the peripheral blood of HIV seropositive individuals included CD4 lymphocytes, monocytes and dendritic cells, all of which express cell surface CD4. However *in vitro* results show a much wider cellular range with evidence for infection of CD4 and CD8 lymphocytes, dendritic cells, monocytes and natural killer cells. In this study CD4 and CD8 lymphocytes, monocytes, B cells, dendritic cells and natural killer cells were isolated from the peripheral blood of HIV infected individuals at different stages of disease progression. A limiting dilution nested PCR technique was used to quantify HIV-1 proviral DNA in each cell subset. HIV-1 DNA was detected in CD4 and CD8 lymphocytes, monocytes, dendritic cells and natural killer cells. PCR data and measurements of the relative frequency of each cell subset were used to calculate the relative contributions of the individual cell subset to total viral load. Surprisingly, not only are CD8 lymphocytes infected *in vivo*, they are a major reservoir of HIV-1 within the peripheral blood of individuals with AIDS.

HIV infection of CD4 and CD8 lymphocytes was detected at all stages of disease progression. Monocyte infection was detected only in a few individuals and in these cases the level of infection was consistently low. This supports the theory that differentiation into macrophages is required for *in vivo* infection. In general the

frequency of CD4 lymphocyte and CD8 lymphocyte infection increased with disease progression. However there appeared to be very little correlation between the level of DC or monocyte infection with mean CD4 counts. A RT-PCR based method was developed to detect HIV-1 mRNA transcripts in different cell subsets for which proviral loads had previously been determined. HIV-1 *vpu-env* and *vpr* mRNA transcripts were detected in CD4 and CD8 lymphocytes and at a low level in monocytes isolated from the peripheral blood of seropositive individuals. This finding suggests active replication of HIV in the mature CD8 lymphocyte population *in vivo* even although they do not express CD4. These findings rule out the possibility that CD8 lymphocyte infection is defective or that transcription is inhibited in this cell type.

Sequence comparisons of the V3 region of the envelope gene indicated that in some individuals, different virus populations may be present in CD4 and CD8 lymphocytes. This provides further independent evidence for HIV infection of CD8 lymphocytes. V3 amino acid sequences showed restricted variation and this was evident irrespective of disease status and cell type. In the main the amino acid sequences were indicative of a NSI-macrophage tropic *in vitro* phenotype.

Recent studies have shown by virus quantitation and mutation fixation rates that the rate of CD4 lymphocyte turnover is close to the number of lymphocytes expressing HIV-1 mRNA in the body (Ho *et al.,* 1995; Wei *et al.,* 1995), suggesting that HIV-1 infection involves continual rounds of viral replication, cell destruction and cell renewal. Rather than immune dysfunction or secondary to CD4

lymphocyte loss, the immunodeficiency observed in AIDS may be a direct result of the cytopathic effect of active HIV-1 infection. In AIDS a reduction in the cell numbers and function of CD4 lymphocytes, CD8 lymphocytes, dendritic cells and natural killer cells is observed. These were the principal cell types infected within the peripheral blood of HIV seropositive individuals. CD4 and CD8 naive lymphocytes are lost preferentially as total CD4 counts fall. It may be that the stimulation of naive cells makes these cells susceptible to HIV infection and destruction. HIV-1 DNA detected in memory cells may represent differentiated naive cells with a defective infection. Preferential naive cell depletion may contribute greatly to the immunodeficiency associated with HIV-1 as these cells are required for all new T cell mediated immune responses and are memory cell precursors.

This study provides evidence for the widespread infection of CD4-negative cells within the peripheral blood of HIV seropositive individuals, indicating that HIV has a broader tropism for cell types *in vivo* than described previously. Active infection of CD4 and CD8 lymphocytes, dendritic cells and natural killer cells may provide novel mechanisms for the immunodeficiency associated with HIV-1 infection.

6.2 Prospects

This study has given rise to many potentially interesting investigations. Clearly, it would be useful to determine the possible mechanisms for HIV-1 infection of CD4-negative cells. Initial studies reported here show that CD8 lymphocytes up-regulate CD4 expression when stimulated with PHA. In order to understand this phenomenon better CD8 lymphoctes should be stimulated under more physiological conditions with for example anti-CD3. It is possible that stimulation of CD8 T cells *in vivo* may up-regulate CD4 expression allowing infection with HIV. Another possible mechanism for HIV-1 infection of CD4-negative cells is using a second receptor, CCR5 or CXCR4 for example. A panel of monoclonal antibodies against various cell markers including the chemokine receptors implicated in HIV infection conjugated to flourescent dyes could be used for immunocytochemistry or FACS analysis of PBMCs. Determination of which cell subsets express chemokine receptors will be useful in investigating there role in infection of CD4-negative cells. A mRNA RT-PCR technique with primers complementary to nucleotide sequences of each of the chemokine receptors reported to be involved in HIV infection would also help in determining the cellular expession of these receptors.

Naive and memory CD4 and CD8 lymphocytes should be isolated from the peripheral blood of HIV-1 seropositive individuals. PCR for HIV-1 DNA and mRNA may give some insight into the reasons why naive cells are preferentially depleted during HIV infection. It will be important to monitor serial samples from

individuals before and during anti-retroviral therapy. The frequency of infection of different cell types and cell counts could be compared as could the emergence of resistant mutants in each cell type. This would give a greater insight into cell and virus turnover rates in each cell subset.

Investigations of cell death, syncytium formation and functional assays for CTL responses in CD8 lymphocytes from HIV-1 positive cultures and negative controls will give an insight into the importance of HIV-1 infection of this cell type and immune dysfuction. Similiar studies using monocytes and natural killer cells would also help to determine the role of HIV-1 infection in cell loss.

Natural killer cells do not express CD4 or mRNA for CD4. Investigations of proviral load and HIV-1 mRNA expression in natural killer cells would be useful in determining whether *in vivo* infection and virus replication occurs in CD4 negative cells.

Bibliography

ALKHATIB, G., COMBADIERE, C., BRODER, C.C., FENG, Y., KENNEDY, P.E., MURPHY, P.M. & BERGER, E.A. (1996). CC CKR5: A RANTES, MIP-1 alpha, MIP-1 beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955-1958.

ANDEWEG, A.C., LEEFLANG, P., OSTERHAUS, A.D.M.E. & BOSCH, M.L. (1993). Both the V2 and V3 regions of the human immunodeficiency virus type-1 surface glycoprotein functionally interact with other envelope regions in syncytium formation. *J.Virol.* **67**, 3232-3239.

ARENS, M., JOSEPH, T., NAG, S., MILLER, J.P., POWDERLY, W.G. & RATNER, L. (1993). Alterations in spliced and unspliced HIV-1-specific RNA detection in peripheral blood mononuclear cells of individuals with varying CD4-positive lymphocyte counts. *AIDS Res.Hum.Retroviruses* **9**, 1257-1263.

BACHELDER, R.E. & LETVIN, N.L. (1996). Postbinding functions of CD4 in HIV infection. *Trends in Microbiology*

BAGASRA, O. & POMERANTZ, R.J. (1993). Human immunodeficiency virus type I provirus is demonstrated in peripheral blood monocytes in vivo - a study utilizing an in situ polymerase chain reaction. *AIDS Res.Hum.Retroviruses* **9**, 69-76.

BARRE-SINOUSSI, F., CHERMAN, J.C., REY, F., NUGEYRE, M.T., CHAMARET, S., GRUEST, J., DAUGUET, C. & AXLER-BLIN, C. (1983). Isolation of a T lymphotropic retrovirus from a patient at risk for aquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871.

BELL, J.E., BUSUTTIL, A., IRONSIDE, J.W., REBUS, S., DONALDSON, Y.K., SIMMONDS, P. & PEUTHERER, J.F. (1993). Human immunodeficiency virus and the brain - investigation of virus load and neuropathologic changes in pre-AIDS subjects. *Journal of Infectious Diseases* **168**, 818-824.

BERKOWER, I., MURPHY, D., SMITH, C.C. & SMITH, G.E. (1991). A predominant group-specific neutralizing epitope of human immunodeficiency virus type 1 maps to residues 342 to 511 of the envelope glycoprotein gp120. *J.Virol.* **65**, 5983-5990.

BERSON, J.F., LONG, D., DORANZ, B.J., RUCKER, J., JIRIK, F.R. & DOMS, R.W. (1996). A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains. *J.Virol.* **70**, 6288-6295.

BINLEY, J., & MOORE, J.P. (1997). HIV-cell fusion. The viral mousetrap. *Nature,* **387 (6631)**, 346-8.

BOUR, S., GELEZIUNAS, R. & WAINBERG, M.A. (1995). The Human Immunodeficiency Virus Type 1 (HIV-1) CD4 Receptor and Its Central Role in Promotion of HIV Infection. *Microbiol.Rev.* **59**, 63-85.

BOYD, M.T., SIMPSON, G.R., CANN, A.J., JOHNSON, M.A. & WEISS, R.A. (1993). A single amino acid substitution in the V1 loop of human immunodeficiency virus type 1 gp120 alters cellular tropism. *J.Virol.* **67**, 3649-3652.

BRENNER, B.G., DASCAL, A., MARGOLESE, R.G. & WAINBERG, M.A. (1997). Natural killer cell function in patients with acquired immunodeficiency syndrome and related diseases. *J. Leukocyte Biol.* **46**, 75-83.

BUKRINSKAYA, A.G., GHORPADE, A., HEINZINGER, N.K., SMITHGALL, T.E., LEWIS, R.E. & STEVENSON, M. (1996). Phosphorylation dependent human immunodeficiency type 1 infection and nuclear targeting of viral DNA. *Proc. Natl. Acad. Sci. USA.* **93 (1).** 367-71.

BUKRINSKY, M.I., SHAROVA, N., DEMPSEY, M.P., STANWICK., T.L., BUKRINSKAYA A.G., HAGGERTY, S., & STEVENSON, M. (1992). Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl. Acad. Sci. USA.* **89,** 6580-6584.

BUKRINSKY, M.I., HAGGERTY, S., DEMPSEY, M.P., SHAROVA, N., ADZHUBEL, A., SPITZ, L., LEWIS, P., GOLFARB, D., EMERMAN, M. & STEVENSON, M. (1993). A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature.* **365 (6447)**, 666-9.

BUKRINSKY, M.I., & HAFFAR O.M. (1997). HIV-1 nuclear import: In search of a leader. *Fronteirs in Bioscience,* **2**, 578-87.

CALABRO, M.L., ZANOTTO, C., CALDERAZZO, F., CRIVELLARO, C., DELMISTRO, A., DEROSSI, A. & CHIECOBIANCHI, L. (1995). HIV-1 infection of the thymus: evidence for a cytopathic and thymotropic viral variant in vivo. *AIDS Res.Hum.Retroviruses* **11**, 11-19.

CAMERON, P.U., FREUDENTHAL, P.S., BARKER, J.M., GEZELTER, S., INABA, K. & STEINMAN, R.M. (1992). Dendritic cells exposed to human immunodeficiency virus type 1 (HIV-1) transmit a vigorous cytopathic effect to CD4+ T cells. *Science* **257**, 383-387.

CAMERON, P.U., POPE, M., GEZELTER, S. & STEINMAN, R.M. (1997). Infection and Apoptotic Cell Death of CD4+ T Cells during an Immune Response to HIV-1 Pulsed Dendritic Cells. *AIDS Res Hum Retroviruses* **10**, 61-71.

CARBONARI, M., CIBATI, M., PESCE, A.M., SBARIGIA, D., GROSSI, P., DOFFIZI, G., LUZI, G. & FIORILLI, M. (1995). Frequency of provirus-bearing CD4(+) cells in HIV type 1 infection correlates with extent of in vitro apoptosis of CD8(+) but not of CD4(+) cells. *AIDS Res.Hum.Retroviruses* **11**, 789-794.

CAUX, C., LIU, Y.J. & BANCHEREAU, J. (1995). Recent advances in the study of dendritic cells and follicular dendritic cells. *Immunology today* **16**, 2-4.

CHAN, D.C., FASS, D., BERGER, J.M., & KIM P.S., (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell*, **89 (2)**, 263-73.

CHEHIMI, J., BANDYOPADHYAY, S., PRAKASH, K., PERUSSIA, B., HASSAN, N.F., KAWASHIMA, H., CAMPBELL, D., KORNBLUTH, J. & STARR, S.E. (1991). In vitro infection of natural killer cells with different human immunodeficiency virus type 1 isolates. *J.Virol.* **65**, 1812-1822.

CHEHIMI, J., PRAKASH, K., SHANMUGAM, V., COLLMAN, R., JACKSON, S.J., BANDYOPADHYAY, S. & STARR, S.E. (1993). CD4-independent infection of human peripheral blood dendritic cells with isolates of human immunodeficiency virus type-1. *J.Gen.Virol.* **74**, 1277-1285.

CHENG-MAYER, C., WEISS, C., SETO, D. & LEVY, J.A. (1989). Isolates of human immunodeficiency virus type 1 from brain may constitute a special group of the AIDS virus. *Proc.Natl.Acad.Sci.U.S.A.* **86**, 8575-8579.

CHESEBRO, B., WEHRLY, K., NISHIO, J. & PERRYMAN, S. (1992). Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparsion with T cell-tropic isolates: definition of critical amino acids involved in cell tropism. *J.Virol* **66**, 6547-6554.

CHOE, H., FARZAN, M., SUN, Y., SULLIVAN, N., ROLLINS, B., PONATH, P.D., WU, L.J., MACKAY, C.R., LAROSA, G., NEWMAN, W., GERARD, N., GERARD, C. & SODROSKI, J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135-1148.

CLERICI, M., SARIN, A., COFFMAN, R.L., WYNN, T.A., BLATT, S.P., HENDRIX, C.W., WOLF, S.F., SHEARER, G.M. & HENKART, P.A. (1994). Type 1/type 2 cytokine modulation of t-cell programed cell death as a model for human immunodeficiency virus pathogenesis. *Proc.Natl.Acad.Sci.USA* **91**, 11811-11815.

CLERICI, M. & SHEARER, G.M. (1997). The Th1-Th2 hypothesis of HIV infection: new insights. *Immunology today* **15**, 575-581.

COCCHI, F., DEVICO, A.L., GARZINO-DEMO, A., CARA, A., GALLO, R.C. & LUSSO, P. (1996). The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. *Nature Med* **1**, 1244-1247.

COFFIN, J. M. (1996). Retroviridae: The viruses and their replication. In *Fields Virology*, pp. 1767-1848. Edited by B. N. Feilds, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman & S. E. Straus. Philadelphia: Lippincott-Raven.

COFFIN, J.M. (1992). Genetic diversity and evolution of retroviruses. *Curr.Top.Microbiol.Immunol.* **176**, 143-164.

COLLMAN, R., GODFREY, B., CUTILLI, J., RHODES, A., HASSAN, N.F., SWEET, R., DOUGLAS, S.D., FRIEDMAN, H., NATHANSON, N. & GONZALEZ-SCARANO, F. (1990). Macrophage-tropic strains of human immunodeficiency virus type 1 utilize the CD4 receptor. *J.Virol.* **64**, 4468-4476.

CONNOR, R.I. & HO, D.D. (1994). Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J.Virol.* **68**, 4400-4408.

DALGLEISH, A.G., BEVERLY, P,C., CLAPHAM, P.R., CRAWFORD, D.H., GREAVES, M.F., WEISS, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature,* **312,** 763-67.

DANIEL, M.D., LETVIN, N.L. & KING, N.W. (1985). Isolation of T-cell tropic HTLV-III-like retrovirus from macaques. *Science* **228**, 1201-1204.

DE JONG, J.J., DE RONDE, A., KEULEN, W., TERSMETTE, M. & GOUDSMIT, J. (1992). Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. *J.Virol.* **66**, 6777-6780.

DE MARIA, A., PANTALEO, G., SCHNITTMAN, S.M., GREENHOUSE, J.J., BASELER, M., ORENSTEIN, J.M. & FAUCI, A.S. (1991). Infection of CD8+ T lymphocytes with HIV. Requirements for interaction with infected CD4+ cells and induction of infectious virus from chronically infected CD8+ cells. *J.Immunol* **146**, 2220-2226.

DE MARIA, A., COLOMBINI, S., SCHNITTMAN, S. & MORETTA, L. (1994). CD8+ cytolytic T lymphocytes become infected in vitro in the process of killing HIV-1-infected target cells. *Eur. J. Imunol.* **24**, 531-536.

DEAN, G.A., REUBEL, G.H. & PEDERSEN, N.C. (1996). Simian immunodeficiency virus infection of CD8+ lymphocytes in vivo. *J.Virol* **70**, 5646-5650.

DENG, H.K., LIU, R., ELLMEIER, W., CHOE, S., UNUTMAZ, D., BURKHART, M., DIMARZIO, P., MARMON, S., SUTTON, R.E., HILL, C.M., DAVIS, C.B., PEIPER, S.C., SCHALL, T.J., LITTMAN, D.R. & LANDAU, N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661-666.

DONALDSON, Y.K., BELL, J.E., HOLMES, E.C., HUGHES, E.S., BROWN, H.K. & SIMMONDS, P. (1994a). In vivo distribution and cytopathology of variants of human immunodeficiency virus type 1 showing restricted sequence variability in the V3 loop. *J.Virol.* **68**, 5991-6005.

DONALDSON, Y.K., BELL, J.E., IRONSIDE, J.W., BRETTLE, R.P., ROBERTSON, J.R., BUSUTTIL, A. & SIMMONDS, P. (1994b). Redistribution of HIV outside the lymphoid system with onset of AIDS. *Lancet* **343**, 382-385.

DORANZ, B.J., RUCKER, J., YI, Y.J., SMYTH, R.J., SAMSON, M., PEIPER, S.C., PARMENTIER, M., COLLMAN, R.G. & DOMS, R.W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149-1158.

DRAGIC, T., LITWIN, V., ALLAWAY, G.P., MARTIN, S.R., HUANG, Y.X., NAGASHIMA, K.A., CAYANAN, C., MADDON, P.J., KOUP, R.A., MOORE, J.P. & PAXTON, W.A. (1996). HIV-1 entry into CD4(+) cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667-673.

EMBRETSON, J., ZUPANCIC, M., RIBAS, J.L., BURKE, A., RACZ, P., TENNERRACZ, K. & HAASE, A.T. (1993). Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* **362**, 359-362.

FENG, Y., BRODER, C.C., KENNEDY, P.E. & BERGER, E.A. (1996). HIV-1 entry cofactor: Functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-877.

FENYO, E.M., MORFELDT MANSON, L., CHIODI, F., LIND, B., VON GEGERFELT, A., ALBERT, J., OLAUSSON, E. & ASJO, B. (1988). Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates. *J.Virol.* **62**, 4414-4419.

FINBLOOM, D.S., HOOVER, D.L. & MELTZER, M.S. (1991). Binding of recombinant HIV coat protein gp120 to human monocytes. *J. Immunol.* **146**, 1316-1321.

FINKEL, T.H., TUDOR WILLIAMS, G., BANDA, N.K., COTTON, M.F., CURIEL, T., MONKS, C., BABA, T.W., RUPRECHT, R.M. & KUPFER, A. (1995). Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nature Med* **1**, 129-134.

FLAMAND, L., CROWLEY, R.W., LUSSO, P., COLOMBINI-HATCH, S., MARGOLIS, D. & GALLO, R.C. (1997). CD4-mediated infection of primary CD8+ lymphocytes by human immunodeficienc virus. *in press*

FOUCHIER, R.A.M., GROENINK, M., KOOTSTRA, N.A., TERSMETTE, M., HUISMAN, H.G., MIEDEMA, F. & SCHUITEMAKER, H. (1992). Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J.Virol* **66**, 3138-3187.

FREUDENTHAL, P.S. & STEINMAN, R.M. (1990). The distinct surface of dendritic cells, as observed after an improved isolation method. *Proc.Natl.Acad.Sci.USA* **87**, 7698-7702.

FROST, S.D.W. & MICHIE, C. (1996). Lymphocyte dynamics, apoptosis and HIV infection. *Trends in Microbiology* **4**, 77-81.

GALLO, R.C., SALAHUDDIN, S.Z., POPOVIC, M., SHEARER, G.M., KAPLAN, M., HAYNES, B.F., PALKER, T.J., REDFIELD, R., OLESKE, J., SAFAI, B. & ET AL. (1984). Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science.* **224**, 500-503.

GALLAY, P., SWINGLER, S., AIKEN, C. & TRONO, D. (1995). HIV-1 infection of non-dividing cells: C-terminal tyrosine phosphorylation of the viral matrix protein is a key regulator. *Cell* **80 (3),** 379-88.

GALLAY, P., SWINGLER, S., SONG, J., BUSHMAN, F., & TRONO, D. (1995a). HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83 (4),** 569-76.

GALLAY, P., HOPE, T., CHIN, D., & TRONO, D. (1995b). HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc. Natl. Acad. Sci, USA.* **95,** 9825-9830.

GALLAY, P., STITT, V., MUNDY, E., OETTINGER, M., & TRONO, D. (1996). Role of the karyopherin pathway in HIV-1 nuclear import. *J. Virol.* **70 (2),** 1027-32.

GOLDFARB, D.S. (1995). HIV-1 virology. Simply marvelous nuclear transport. *Curr. Biol.* **5 (6)**, 570-3.

GOUGEON, M.L., LECOEUR, H., DULIOUST, A., ENOUF, M.G., CROUVOISIER, M., GOUJARD, C., DEBORD, T. & MONTAGNIER, L. (1996). Programmed cell death in peripheral lymphocytes from HIV-infected persons - Increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. *J.Immunol* **156**, 3509-3520.

GUNTAKA, R.V. (1993). Transcription termination and polyadenylation in retroviruses. *Microbiol.Rev.* **57**, 511-521.

HARPER, M.E., MARSELLE, L.M., GALLO, R.C. & WONG STAAL, F. (1986). Detection of lymphocytes expressing human T lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridisation. *Proc.Natl.Acad.Sci.USA* **83**, 772-776.

HIRSCH, M. S. & CURRAN, J. W. (1996). Human Immunodeficiency viruses. In *Fields Virology*, pp. 1953-1976. Edited by B. N. Feilds, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman & S. E. Straus. Philadelphia: Lippincott-Raven.

HO, D.D., NEUMANN, A.U., PERELSON, A.S., CHEN, W., LEONARD, J.M. & MARKOWITZ, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123-126.

HO, D.D. (1997). Advances painted in shades of gray at a DC conference. *Science* **275**, 31

HOLMES, E.C., ZHANG, L.Q., SIMMONDS, P., LUDLAM, C.A. & LEIGH BROWN, A.J. (1992). Convergent and divergent sequence evolution in the surface envelope glycoprotein of human immunodeficiency virus type 1 within a single infected patient. *Proc.Natl.Acad.Sci.U.S.A.* **89**, 4835-4839.

HSIA, K. & SPECTOR, S.A. (1991). Human immunodeficiency virus DNA is present in a high percentage of CD4+ lymphocytes of seropositive individuals. *J Infect Dis* **164**, 470-475.

HSIA, K., TSAI, V., ZVAIFLER, N.J. & SPECTOR, S.A. (1995). Low prevalence of HIV-1 proviral DNA in peripheral blood monocytes and dendritic cells from HIV-1-infected individuals. *AIDS* **9**, 398-399.

HWANG, S.S., BOYLE, T.J., LYERLY, H.K. & CULLEN, B.R. (1991). Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**, 71-74.

INNOCENTI, P., OTTMANN, M., LECLERCQ, P. & SEIGNEURIN, J.M. (1992). HIV-1 in blood monocytes: frequency of detection of proviral DNA using PCR and comparison with total CD4 count. *AIDS Res.Hum.Retroviruses* **8**, 261-268.

JOHNSON, R.P. & WALKER, B.D. (1994). Cytotoxic T lymphocytes in Human Immunodeficiency Virus Infection: Response to Structural Proteins. *Current Topics in Microbiology and Immunology* **189**, 35-63.

KITCHEN, S.J., UITTENBOGAART, C.H. & ZACK, J. (1997). Mechanisms of HIV-1 localisation in CD4-negative thymocytes: Differentiation from a CD4-positive precursor allows productive infection. *J Virol* **71**, 5713-5722.

KLAZMANN, D., CHAMPAGNE, E., CHAMARET, S., *et al*, (1984). T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature*, **312,** 767-68.

KNIGHT, S.C. (1994). Infection of dendritic cells with HIV type 1. *AIDS Res.Hum.Retroviruses* **10**, 1591-1592.

KNIGHT, S.C. (1996). Bone-marrow-derived dendritic cells and the pathogenesis of AIDS. *AIDS* **10**, 807-817.

KUIKEN, C.L., DE JONG, J., BAAN, E., KEULEN, W., TERSMETTE, M. & GOUDSMIT, J. (1992). Evolution of the V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during transition of the viral biological phenotype. *J.Virol.* **66**, 4622-4627.

KUMAR, S., TAMURA, K. & NEI, M. (1993). In *MEGA: Molecular Evolutionary Genetics Analysis, version 1.0.* University Park. The Pensylvannia State University.

KUSUMI, K., CONWAY, B., CUNNINGHAM, S., BERSON, A., EVANS, C., IVERSEN, A.K., COLVIN, D., GALLO, M.V., COUTRE, S., SHPAER, E.G. & ET AL. (1992). Human immunodeficiency virus type 1 envelope gene structure and diversity in vivo and after cocultivation in vitro. *J.Virol.* **66**, 875-885.

LANE, H.C., MASUR, H., EDGAR, L.C., WHALEN, G., ROOK, A.H. & FAUCI, A.S. (1983). Abnormalities of B cell activation and immunoregulation in patients with acquired immunodeficiency syndrome. *N.Engl.J.Med* **309**, 453-458.

LANGHOFF, E., KALLAND, K.H. & HASELTINE, W.A. (1993). Early molecular replication of human immunodeficiency virus type-1 in cultured-blood-derived t-helper dendritic cells. *Journal of Clinical Investigation* **91**, 2721-2726.

LAROSA, G.J., DAVIDE, J.P., WEINHOLD, K., WATERBURY, J.A., PROFY, A.T., LEWIS, J.A., LANGLOIS, A.J., DREESMAN, G.R., BOSWELL, R.N. & SHADDUCK, P. (1990). Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. *Science* **249**, 932-935.

LEVY, J.A. (1993). Pathogenesis of human immunodeficiency virus infection. *Microbiol.Rev.* **57**, 183-289.

LEVY, J.A., MACKEWICZ, C.E. & BARKER, E. (1996). Controlling HIV pathogenisis: the role of the noncytotoxic anti-HIV response of CD8+ T cells. *Immunology today* **17**, 217-224.

LEWIN, S.R., SONZA, S., IRVING, L.B., MCDONALD, C.F., MILLS, J. & CROWE, S.M. (1996). Surface CD4 is critical to in vitro HIV infection of human alveolar macrophages. *AIDS Res.Hum.Retroviruses* **12**, 877-883.

LOUWAGIE, J., MCCUTCHAN, F.E., PEETERS, M., BRENNAN, T.P., SANDERSBUELL, E., EDDY, G.A., VANDERGROEN, G., FRANSEN, K., GERSHYDAMET, G.M., DELEYS, R. & BURKE, D.S. (1993). Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. *AIDS* **7**, 769-780.

LUCIW, P. (1996). Human immunodeficiency viruses and their replication. In *Fields Virology*, pp. 1881-1952. Edited by B. N. Feilds, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman & S. E. Straus. Philadelphia: Lippincott-Raven.

LUNDBERG, K., HEATH, W., KONTGEN, F., CARBONE, F.R. & SHORTMAN, K. (1995). Intermediate steps in positive selection: Differentiation of CD4+CD8 int Thymocytes into CD4-8+ TCR hi Thymocytes. *J Exp Med* **181**, 1643-1651.

MACATONIA, S.E., LAU, R., PATTERSON, S., PINCHING, A.J. & KNIGHT, S.C. (1990). Dendritic cell infection, depletion and dysfunction in HIV-infected individuals. *Immunology* **71**, 38-45.

MADDON, P.J., DALGLEISH, A.G., McDOUGAL, J.S., CLAPHAM, P.R., WEISS, R.A., AXEL, R. (1986). The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell,* **47**, 333-48.

MAHALINGARN, S., AYYAVOO, V., PATEL, M., KIEBER EMMONS, T., & WEINER, D.B. (1997). Nuclear import, virion incorporation, and cell cycle arrest/differentiation are mediated by distinct functional domains of human immunodeficiency virus type 1 Vpr. *J. Virol.* **71 (9)**, 6339-47.

MARGOLICK, J.B., MUNOZ, A., DONNENBERG, A.D., PARK, L.P., GALAI, N., GIORGI, J.V., O'GORMAN, M.R.G. & FERBAS, J. (1997). Failure of T-cell homeostasis preceding AIDS in HIV infection. *Nature Med* **1**, 674-680.

MASON, H. (1997). In *Low levels of CD4, CD8 double positve lymphocytes in the peripheral blood of seropositive individuals.* (UnPub)

MASUDA, T., MATSUSHITA, S., KURODA, M.J., KANNAGI, M., TAKATSUKI, K. & HARADA, S. (1990). Generation of neutralization-resistant HIV-1 in vitro due to amino acid interchanges of third hypervariable env region. *J.Immunol.* **145**, 3240-3246.

MCILROY, D., AUTRAN, B., CHEYNIER, R., WAINHOBSON, S., CLAUVEL, J.P., OKSENHENDLER, E., DEBRE, P. & HOSSMALIN, A. (1995). Infection frequency of dendritic cells and CD4(+) t lymphocytes in spleens of human immunodeficiency virus- positive patients. *J.Virol.* **69**, 4737-4745.

MCILROY, D., AUTRAN, B., CHEYNIER, R., CLAUVEL, J.P., OKSENHENDLER, E., DEBRE, P. & HOSMALIN, A. (1996). Low infection frequency of macrophages in the spleens of HIV+ patients. *Res.Virology* **147**, 115-121.

MERCURE, L., PHANEUF, D. & WAINBERG, M.A. (1993). Detection of unintegrated human immunodeficiency virus type-1 DNA in persistently infected CD8+ cells. *J.Gen.Virol.* **74**, 2077-2083.

MEYERHANS, A., CHEYNIER, R., ALBERT, J., SETH, M., KWOK, S., SNINSKY, J., MORFELDT MANSON, L., ASJO, B. & WAIN HOBSON, S. (1989). Temporal fluctuations in HIV quasispecies in vivo are not reflected by sequential HIV isolations. *Cell* **58**, 901-910.

MICHAEL, N.L., MO, T., MERZOUKI, A., O'SHAUGHNESSY, M., OSTER, C., BURKE, D.S., REDFIELD, R.R., BIRX, D.L. & CASSOL, S.A. (1995). Human immunodeficiency virus type 1 cellular RNA load and splicing patterns predict disease progression in a longitudinally studied cohort. *J Virol* **69**, 1868-1877.

MILICH, L., MARGOLIN, B. & SWANSTROM, R. (1993). V3 loop of the human immunodeficiency virus type-1 env protein -interpreting sequence variability. *J.Virol.* **67**, 5623-5634.

MITCHELL, W.M., FORTI, R.L., VOGLER, L.B., LAWTON, A.R. & GREGG, C.R. (1994). Spontaneous and interferon resistant natural killer cell anergy in AIDS. *AIDS Res.* **1**, 221-229.

MYERS, G., KORBER, B., BERZOFSKY, J., SMITH, T. F. & PAVLAKIS, G. N. (1991). In *Human retroviruses and AIDS 1993*, Los Alamos, New Mexico: Los Alamos National Laboratory.

NEUMANN, M., HARRISON, J., SALTARELLI, M., HADZIYANNIS, E., ERFLE, V., FELBER, B.K. & PAVLAKIS, G.N. (1994). Splicing variability in HIV type 1 revealed by quantitative polymerase chain reaction. *AIDS Res.Hum.Retroviruses* **10**, 1531-1542.

O'DOHERTY, U., STEINMAN, R.M., PENG, M., CAMERON, P.U., GEZELTER, S., KOPELOFF, I., SWIGGARD, W.J., POPE, M. & BHARDWAJ, N. (1993). Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J.Exp.Med.* **178**, 1067-1076.

OBERLIN, E., AMARA, A., BACHELERIE, F., BESSIA, C., VIRELIZIER, J.L., ARENZANASEISDEDOS, F., SCHWARTZ, O., HEARD, J.M., CLARKLEWIS, I., LEGLER, D.F., LOETSCHER, M., BAGGIOLINI, M. & MOSER, B. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833-835.

PANTALEO, G., GRAZIOSI, C. & FAUCI, A.S. (1993). Mechanisms of disease-the immunopathogenesis of human immunodeficiency virus infection. *N.Engl.J.Med.* **328**, 327-335.

PATTERSON, S., ROBERTS, M.S., ENGLISH, N.R., MACATONIA, S.E., GOMPELS, M.N., PINCHING, A.J. & KNIGHT, S.C. (1994). Detection of HIV DNA in peripheral blood dendritic cells of HIV-infected individuals. *Res.Virology* **145**, 171-176.

PETERS, J.P., GIESLER, R., THIELE, B. & STEINBACH, F. (1996). Dendritic cells: from ontogenetic orphans to myelomonocytic descendants. *Immunology today* **17**, 273-278.

PREMACK, B.A. & SCHALL, T.J. (1996). Chemokine receptors: Gateways to inflammatiom and infection. *Nature Med* **2**, 1174-1178.

PSALLIDOPOULOS, M.C., SCHNITTMAN, S.M., THOMPSON, L.M., BASELER, M., FAUCI, A.S., LANE, H.C. & SALZMAN, N.P. (1989). Integrated proviral human immunodeficiency virus type 1 is present in CD4+ peripheral lymphocytes in healthy seropositive individuals. *J.Virol.* **63**, 4626-4631.

PURCELL, D.F.J. & MARTIN, M.A. (1993). Alternative splicing of human immunodeficiency virus type- 1 messenger RNA modulates viral protein expression, replication, and infectivity. *J.Virol.* **67**, 6365-6378.

RABIN, R.L., ROEDERER, M., MALDONADO, Y., PETRU, A. & HERZENBERG, L.A. (1995). Altered representation of naive and memory CD8 t cell subsets in HIV-infected children. *Journal of Clinical Investigation* **95**, 2054-2060.

RIBRAG, V., SALMON, D., PICARD, F., GUESNU, M., SICARD, D. & DREYFUS, F. (1993). Increase in double-positive CD4+CD8+ peripheral t-cell subsets in an HIV-infected patient. *AIDS* **7**, 1530

ROBERTS, M.S., GOMPELS, M., PINCHING, A.J. & KNIGHT, S.C. (1994). Dendritic cells persistently stimulate antibody responses to HIV in seropositive individuals (short communication). *AIDS* **8**, 1097-1101.

ROEDERER, M., DUBS, J.G., ANDERSON, M.T., RAJU, P.A. & HERZENBERG, L.A. (1995). CD8 naive t cell counts decrease progressively in HIV- infected adults. *Journal of Clinical Investigation* **95**, 2061-2066.

SALTARELLI, M.J., HADZIYANNIS, E., HART, C.E., HARRISON, J.V., FELBER, B.K., SPIRA, T.J. & PAVLAKIS, G.N. (1996). Analysis of human immunodeficiency virus type 1 mRNA splicing patterns during disease progression in peripheral blood mononuclear cells from infected individuals. *AIDS Res.Hum.Retroviruses* **12**, 1443-1456.

SAMSON, M., LIBERT, F., DORANZ, B.J., RUCKER, J., LIESNARD, C., FARBER, C.M., SARAGOSTI, S., LAPOUMEROULIE, C., COGNAUX, J., FORCEILLE, C., MUYLDERMANS, G., VERHOFSTEDE, C., BURTONBOY, G., GEORGES, M., IMAI, T., RANA, S., YI, Y.J., SMYTH, R.J., COLLMAN, R.G., DOMS, R.W., VASSART, G. & PARMENTIER, M. (1996). Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**, 722-725.

SATTENTAU, Q.J. & MOORE, J.P. (1991). Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *J.Exp.Med.* **174**, 407-415.

SCHNITTMAN, S.M., PSALLIDOPOULOS, M.C., LANE, H.C., THOMPSON, L., BASELER, M., MASSARI, F., FOX, C.H., SALZMAN, N.P. & FAUCI, A.S. (1989). The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. *Science* **245**, 305-308.

SCHNITTMAN, S.M., GREENHOUSE, J.J., LANE, H.C., PIERCE, P.F. & FAUCI, A.S. (1991). Frequent detection of HIV-1-specific mRNAs in infected individuals suggests ongoing active viral expression in all stages of disease. *AIDS Res.Hum.Retroviruses* **7**, 361-367.

SCHUITEMAKER, H., KOOTSTRA, N.A., DE GOEDE, R.E., DE WOLF, F., MIEDEMA, F. & TERSMETTE, M. (1991). Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture. *J.Virol.* **65**, 356-363.

SCHUITEMAKER, H., KOOT, M., KOOTSTRA, N., DERCKSEN, M.W., GOEDE, R.T.D., STEENWIJK, R., LANGE, J.M.A., SCHATTTENKERK, J.K., MIEDEMA, F. & TERSMETTE, M. (1992). Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T cell-tropic virus populations. *J.Virol* **66**, 1354-1360.

SCHULZEOSTHOFF, K., BEYAERT, R., VANDEVOORDE, V., HAEGEMAN, G. & FIERS, W. (1993). Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effects of TNF. *EMBO J.* **12**, 3095-3104.

SCOTTALGARA, D., VUILLIER, F., CAYOTA, A., RAME, V., GUETARD, D., MONCANY, M.L.J., MARASESCU, M., DAUGUET, C. & DIGHIERO, G. (1993). In vitro non-productive infection of purified natural killer cells by the BRU isolate of the human immunodeficiency virus type-1. *J.Gen.Virol.* **74**, 725-731.

SEMENZATO, G., AGOSTINI, C., OMETTO, L., ZAMBELLO, R., TRENTIN, L., CHIECOBIANCHI, L. & DEROSSI, A. (1995). CD8(+) t lymphocytes in the lung of acquired immunodeficiency syndrome patients harbor human immunodeficiency virus type 1. *Blood* **85**, 2308-2314.

SHIODA, T., LEVY, J.A. & CHENG-MAYER, C. (1992). Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell line and macrophage tropism of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA.* **89**, 9434-9438.

SIMMONDS, P., BALFE, P., LUDLAM, C.A., BISHOP, J.O. & LEIGH BROWN, A.J. (1990a). Analysis of sequence diversity in hypervariable regions of the external glycoprotein of human immunodeficiency virus type 1. *J.Virol.* **64**, 5840-5850.

SIMMONDS, P., BALFE, P., PEUTHERER, J.F., LUDLAM, C.A., BISHOP, J.O. & LEIGH BROWN, A.J. (1990b). Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J.Virol.* **64**, 864-872.

SKOWRON, G. (1996). Antiretroviral chemotherapy in HIV infection. *Medicine.* **2**, 145-148.

SLEASMAN, J.W., ALEIXO, L.F., MORTON, A., SKODASMITH, S. & GOODENOW, M.M. (1996). CD4+ memory T cells are the predominant population of HIV-1-infected lymphocytes in neonates and children. *AIDS* **10**, 1477-1484.

SLEPUSHKIN, V.A., ANDREEV, S.M., SIDOROVA, M.V. & MELIKYAN, G.B. (1992). Investigation of human immunodeficiency virus fusion peptides. Analysis of interrelations between their structure and function. *AIDS Res.Hum.Retroviruses* **8**, 9-18.

SONZA, S., MAERZ, A., UREN, S., VIOLO, A., HUNTER, S., BOYLE, W. & CROWE, S. (1995). Susceptibility of human monocytes to HIV type 1 infection in vitro is not dependent on their level of CD4 expression - short communication. *AIDS Res.Hum.Retroviruses* **11**, 769-776.

SPEARMAN, P., HORTON, R., RATNER, L. & KULI ZADE I. (1997). Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. *J. Virol.* **71 (9),** 6582-92.

SPECK, R.F., WEHRLY, K., PLATT, E.J., ATCHISON, R.E., CHARO, I.F., KABAT, D., CHESEBRO, B. & GOLDSMITH, M.A. (1997). Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptos determined by individual amino acids within the envelope V3 loop. *J. Virol* **71**, 7136-7139.

SPINA, C.A., GUATELLI, J.C. & RICHMAN, D.D. (1995). Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. *J.Virol.* **69**, 2977-2988.

STEWART, S.J., FUJIMOTO, J. & LEVY, R. (1986). Human T lymphocytes and monocytes bear the same Leu-3(T4) antigen. *J. Immunol.* **136**, 3773-3778.

STRAPPE, P. M. (1997). In HIV-1 V3 region sequence changes following in vitro culture. (UnPub)

TERSMETTE, M., DE GOEDE, R.E.Y., AL, B.J.M., WINKEL, I.N., GRUTERS, R.A., CUYPERS, H.T.M., HUISMAN, H.G. & MIEDEMA, F. (1988). Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J.Virol.* **62**, 2026-2032.

TRKOLA, A., DRAGIC, T., ARTHOS, J., BINLEY, J.M., OLSON, W.C., ALLAWAY, G.P., CHENGMAYER, C., ROBINSON, J., MADDON, P.J. & MOORE, J.P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384**, 184-187.

TSUBOTA, H., RINGLER, D.J., KANNAGI, M., KING, N.W., SOLOMON, K.R., MACKEY, J.J., WALSH, D.G. & LETVIN, N.L. (1989). CD8+CD4- lymphocyte lines can harbour the AIDS virus in vitro. *J.Immunol* **143**, 858-863.

VALENTIN, A., LUNDIN, K., PATARROYO, M. & ASJO, B. (1990). The leukocyte adhesion glycoprotein CD18 participates in HIV-1-induced syncytia formation in monocytoid and T cells. *J.Immunol.* **144**, 934-937.

VALENTIN, A., VON GEGERFELT, A., MATSUDA, S., NILSSON, K. & ASJO, B. (1991). In vitro maturation of mononuclear phagocytes and susceptibility to HIV-1 infection. *J.Acquir.Immune.Defic.Syndr.* **4**, 751-759.

VAZEUX, R., LACROIX-CLAUDE, C., BLANCHE, S., CUMONT, M.S., HENIN, D., GRAY, F., BOCCON-GIBOD, L. & TARDIEU, M. (1992). Low levels of HIV replication in the brain tissue of children with severe AIDS encephalopathy. *Am. J. Pathol.* **140**, 137-144.

VON SCHWEDLER, U., KORNBLUTH, R.S. & TRONO, D. (1994). The nuclear localisation signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. *Pro. Natl. Acad. Sci. Usa.* **91 (15)**, 6992-6.

VUILLIER, F., BIANCO, N.E., MONTAGNIER, L. & DIGHIERO, G. (1989). Selective depletion of low density CD8+, CD16+ lymphocytes during HIV infection. *AIDS Res.Hum.Retroviruses* **4**, 121-125.

WATKINS, B.A., DORN, H.H., KELLY, W.B., ARMSTRONG, R.C., POTTS, B.J., MICHAELS, F., KUFTA, C.V. & DUBOIS DALCQ, M. (1990). Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science.* **249**, 549-553.

WEI, X.P., GHOSH, S.K., TAYLOR, M.E., JOHNSON, V.A., EMINI, E.A., DEUTSCH, P., LIFSON, J.D., BONHOEFFER, S., NOWAK, M.A., HAHN, B.H., SAAG, M.S. & SHAW, G.M. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117-122.

WEISS, R.A. (1993). How does HIV cause AIDS. *Science* **260**, 1273-1279.

WEISSENHORN, W., DESSEN A., HARRISON, SC., SKEHEL, J.J., & WILEY, D.D.(1997). **387 (6631)**, 426-30.

WEISSMAN, D., LI, Y.X., ANANWORANICH, J., ZHOU, L.J., ADELSBERGER, J., TEDDER, T.F., BASELER, M. & FAUCI, A.S. (1995). Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1. *Proc.Natl.Acad.Sci.USA* **92**, 826-830.

WOLFS, T.F., ZWART, G., BAKKER, M., VALK, M., KUIKEN, C.L. & GOUDSMIT, J. (1991). Naturally occurring mutations within HIV-1 V3 genomic RNA lead to antigenic variation dependent on a single amino acid substitution. *Virology*. **185**, 195-205.

WOLFS, T.F.W., ZWART, G., BAKKER, M., VALK, M., KUIKEN, C.L. & GOUDSMIT, J. (1991). Naturally occurring mutations within HIV-1 V3 genomic RNA lead to antigenic variation dependent on a single amino acid substitution. *Virology* **185**, 195-205.

WU, L.J., GERARD, N.P., WYATT, R., CHOE, H., PAROLIN, C., RUFFING, N., BORSETTI, A., CARDOSO, A.A., DESJARDIN, E., NEWMAN, W., GERARD, C. & SODROSKI, J. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384**, 179-183.

ZHANG, L.Q., MACKENZIE, P., CLELAND, A., HOLMES, E.C., LEIGH BROWN, A.J. & SIMMONDS, P. (1993). Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection. *J.Virol.* **67**, 3345-3356.

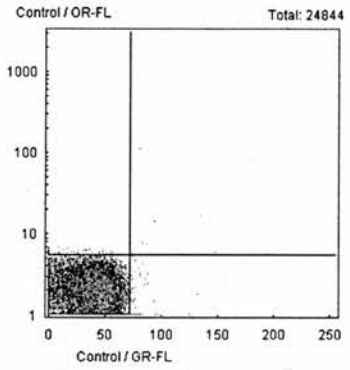
ZWART, G., LANGEDIJK, H., VAN DER HOEK, L., DE JONG, J.J., WOLFS, T.F.W., RAMAUTARSING, C., BAKKER, M., DE RONDE, A. & GOUDSMIT, J. (1991). Immunodominance and antigenic variation of the principal neutralization domain of HIV-1. *Virology* **181**, 481-489.

APPENDIX A : Raw FACS data.

ORTHO TRIO PATIENT SUMMARY REPORT

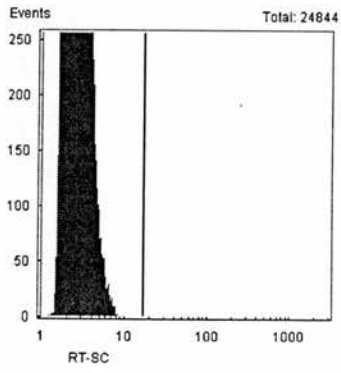
(i) E rosette positive cell fraction isolated from the peripheral blood of an HIV-1 seropositive individual.

Control Antibodies



Graph Number: 2 , Tube: 1
Gated by: A

Reg	%Tot	Events	MeanX	MeanY
--	99.3	24679	34.3	13.5
-+	0.3	70	24.9	56.6
+-	0.4	89	79.8	12.4
++	0.0	6	109.2	83.0
(+*)	0.4	95		
(*+)	0.3	76		

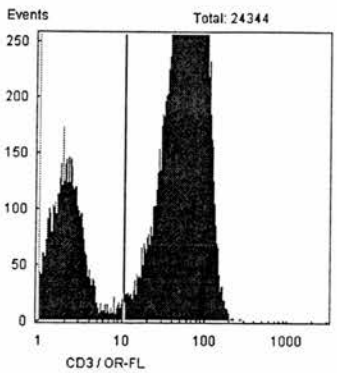


Graph Number: 6 , Tube: 1
Gated by: A

Reg	%Tot	Events	Mean	Peak
1	100.0	24844	28.6	20
2	0.0	0	0.0	0

Reg	SD	CV%
1	9.4	32.9
2	0.0	0.0

CD3

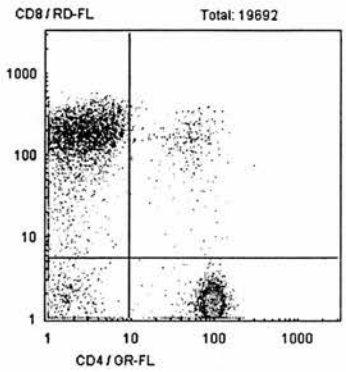


Graph Number: 4 , Tube: 2
Gated by: A

Reg	%Tot	Events	Mean	Peak
1(-)	25.4	6195	20.9	0
2(+)	74.6	18149	129.1	138

Reg	SD	CV%
1(-)	17.2	82.0
2(+)	16.6	12.8

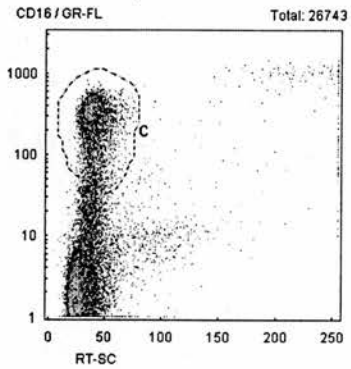
CD4/CD8



Graph Number: 3 , Tube: 2
Gated by: B

Reg	%Tot	Events	MeanX	Mean
--	6.1	1206	11.2	14.2
-+	31.6	6223	23.6	159.1
+-	60.1	11844	145.7	4.9
++	2.1	419	121.3	151.9
(+*)	62.3	12263		
(*+)	33.7	6642		

CD16



Graph Number: 3 , Tube: 3

Reg	%Tot	Events	MeanX	Mean
C	18.1	4851	40.6	173.4

Summary report

Lymphocyte Count (Cells/ul) : 10691 (Based on ImmunoSum)

CD4 Count (Cells/ul) : 5250

CD4/CD8 ratio : 1.9

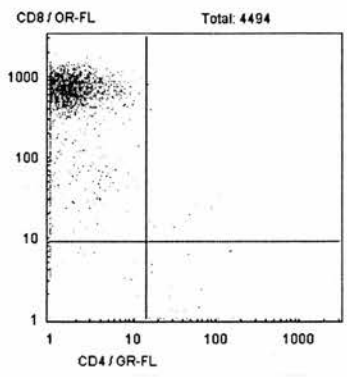
Sample	Cells/ul	% of Lymphs
CD3+ T-cells:	8556	80.0
CD4+ T-cells:	5250	49.1
CD8+ T-cells:	2758	25.8
CD19+ B-cells:	20	0.2
CD16+CD3- NK-cells:	2113	19.8

APPENDIX A : Raw FACS data.

ORTHO TRIO PATIENT SUMMARY REPORT

(ii) Positively selected CD8 cell fraction isolated from the peripheral blood of an HIV-1 seropositive individual using the miniMACS system.

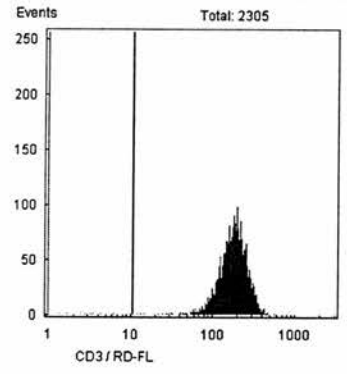
CD8/CD4



FCS: wendy_8.005
Graph Number: 3 , Tube: 5
Gated by: B (CD3RT)

Reg	%Gated	Events	MeanX	Mean
--	0.6	28	26.4	38.9
-+	98.5	4428	10.0	202.0
+-	0.6	26	114.2	15.7
++	0.3	12	113.2	111.9
(+*)	0.8	38		
(*+)	98.8	4440		

CD3



FCS: wendy_8.005
Graph Number: 4 , Tube: 5
Gated by: A (LYMPH)

Reg	%Gated	Events	Mean	Peak
1	0.4	10	34.6	73
2	99.6	2295	165.0	169

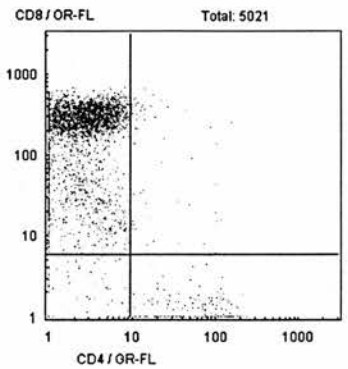
Reg	SD	CV%
1	22.1	64.0
2	12.7	7.7

APPENDIX A : Raw FACS data.

ORTHO TRIO PATIENT SUMMARY REPORT

(iii) Positively selected CD8 cell fraction isolated from the peripheral blood of an HIV-1 seropositive individual using the miniMACS system.

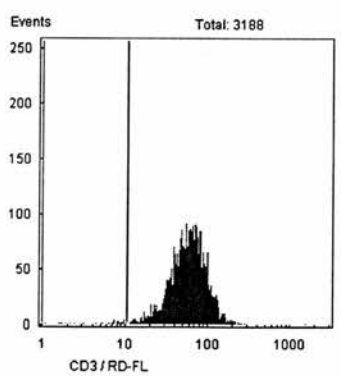
CD8/CD4



FCS: wendy_8.002
Graph Number: 3 , Tube: 5
Gated by: B (CD3RT)

Reg	%Gated	Events	MeanX	Mean
--	1.5	73	31.6	29.4
-+	92.6	4650	24.5	167.6
+-	4.1	208	128.2	7.8
++	1.8	90	91.1	151.4
(+*)	5.9	298		
(*+)	94.4	4740		

CD3



FCS: wendy_8.002
Graph Number: 4 , Tube: 5
Gated by: A (LYMPH)

Reg	%Gated	Events	Mean	Peak
1	1.3	41	53.1	73
2	98.7	3147	129.6	128

Reg	SD	CV%
1	24.0	45.1
2	15.7	12.1

Early reports

Frequent infection of peripheral blood CD8-positive T-lymphocytes with HIV-1

W J Livingstone, M Moore, D Innes, J E Bell, P Simmonds, and the Edinburgh Heterosexual Transmission Study Group

Summary

Background Although lymphocytes expressing the CD4 surface receptor for HIV-1 have been identified as the principal target of the virus, the extent to which infection of other cell types of the immune system contributes to immunodeficiency is unknown. We investigated the cell types in peripheral blood infected with HIV and the relation of viral load in different subsets to disease progression.

Methods The study group consisted of 16 HIV-infected individuals, eight of whom had clinically defined AIDS with CD4 cell counts less than 200/μL blood. The main component subsets of peripheral blood mononuclear cells were purified by magnetic bead separation, and included CD4 and CD8 lymphocytes, B lymphocytes, monocytes, and dendritic cells. HIV proviral sequences within these separate populations were quantified by limiting-dilution nested polymerase chain reaction.

Findings HIV-1 proviral sequences were detected in T-helper cells, cytotoxic T cells, dendritic cells, and monocytes. CD4 T lymphocytes constituted the main reservoir of HIV in all but one of the symptom-free individuals studied (those with CD4 count >200/μL). However, in all the individuals with CD4 counts of less than 200/μL, most infected cells within the peripheral blood mononuclear cell fraction were either dendritic cells or CD8 lymphocytes. Infection of CD8 cells accounted for between 66% and 97% of total proviral load in five of the eight AIDS patients. A strong inverse relation between total CD8 count and the frequency of CD8 T-lymphocyte infection was found.

Interpretation This study provides evidence for widespread infection of lymphocytes of the CD8 phenotype, indicating that HIV-1 has a broader tropism for different cell types in vivo than described for cultured virus. Infection of CD8 cells may contribute to the decline of this subset upon disease progression in HIV-infected individuals. Infection of CD8 cells may or may not occur by a non-CD4-dependent mechanism of virus entry.

Lancet 1996; **348:** 649–54

See Commentary page 631

Introduction

The principal immunological defect occurring with progression of HIV-1 infection is the loss of CD4 T-helper cells, which have a central role in the immune response to pathogens. Theories about the causes of this loss of CD4 cells range from their destruction or dysfunction directly caused by viral infection to apoptosis resulting from defects in antigen presentation.[1] Whether the defects in many immune pathways, including the cytotoxic T-cell response mediated by CD8 lymphocytes, are secondary to the destruction of T-helper lymphocytes is similarly unclear. For example, Macatonia and colleagues suggested that infection of reticulodendritic cells with HIV contributes to the defects in antigen processing and presentation to T-helper cells found in AIDS patients.[2]

In this study, we used standard separation methods to isolate subsets of cells involved in the immune response and thereby to investigate the extent to which each cell type is infected with HIV. It has hitherto been generally accepted that the cellular tropism of HIV is determined almost exclusively by the distribution of CD4, the cell-surface receptor for HIV-1.[3–5] After virus attachment, high-affinity interactions between CD4 and the external envelope glycoprotein (gp120) of HIV-1 initiate conformational changes, resulting in exposure of a fusogenic domain of the transmembrane envelope protein (gp41) which allows entry of the virus into the cytoplasm.[1] CD4 is expressed on T-helper cells, monocytes, and peripheral-blood dendritic cells[6,7] but not on B cells or mature cytotoxic T cells.

Although monocytes express CD4 and the activation marker HLA-DR,[1,8,9] there is little evidence for extensive infection of these cells with HIV. Estimates of the frequency of infected monocytes in the peripheral circulation have ranged from zero to 100 per million of total cells.[10–14] Previous reports have found little evidence for in-vivo infection of mature cytotoxic T cells with HIV,[10,15] although infection of CD8 T lymphocytes has been achieved in vitro.[6]

Peripheral-blood dendritic cells, like monocytes, are a heterogeneous population at different stages of maturation.[6] They are extremely potent antigen-presenting cells and their infection by HIV could be critical in the generation of immune dysfunction. Various groups have isolated populations of these cells that can be infected in vitro[2,16,17] and HIV-1 has been isolated directly from peripheral-blood cells with dendritic-cell characteristics.[14,18]

It is not known whether strains of HIV exist with differences in tropism for different cell types found in peripheral blood mononuclear cell populations. In vitro, macrophage tropism is associated with a non-syncytium-inducing viral (NSI) phenotype. Changes in phenotype and tropism of virus isolates are known to occur during disease progression,[19,20] although no change in the relative frequencies of the different cell types infected has been

Primer	Sequence (5'–3')	Sense (+) or antisense (−)	Position of 5' base in HXB2 genome
V3a	5'-TACAATGTACACATGGAATT-3'	+	6957
V3b	5'-TGGCAGTCTAGCAGAAGAAG-3'	+	7009
V3c	5'-CTGGGTCCCCTCCTGAGG-3'	−	7331
V3d	5'-ATTACAGTAGAAAAATTCCCC-3'	−	7381
gage	5'-GCGAGAGCGTCAGTATTAAGCGG-3'	+	795
gagf	5'-GGGAAAAAATTCGGTTAAGGCC-3'	+	835
gagg	5'-CTTCTACTACTTTTACCCATGC-3'	−	1248
gagh	5'-TCTGATAATGCTGAAAACATGGG-3'	−	1296

Table 1: **Primer sequences**

Department of Medical Microbiology, University of Edinburgh, Edinburgh EH8 9AG, UK (W J Livingstone BSc, P Simmonds MRCPath); **Institute of Cell, Animal and Population Biology, Edinburgh** (M Moore PhD, D Innes BSc); **and Department of Neuropathology, University of Edinburgh, Western General Hospital, Edinburgh** (J E Bell FRCPath)

Correspondence to: Dr Peter Simmonds

Subset removed	Monoclonal antibody	Cell phenotype				
ER positive*		**CD3**	**CD4**	**CD8**	**CD16**	**CD19**
E positive	None	82·8	47·0	40·1	15·5	0·9
CD4 T cells	CD4	54·4	1·7	54·5	14·5	ND
CD8 T cells	CD8	12·0	4·4	4·2	12·7	ND
ER negative†		**CD3**	**CD4**	**CD14**	**CD21**	**HLA-DR**
E negative	None	1·0	64·7	59·5	8·6	ND
Monocytes	CD11b/CD14	1·0	37·8	3·7	ND	ND
B cells	CD19/CD21	1·0	43·5	3·7	ND	53·2

ND=not done. *ER-positive=T cells; mean frequencies of three separate purifications (or two for CD8-depleted cells). †ER -negative=non-T cells (B cells, monocytes, and dendritic cells); results from a single sample.

Table 2: **Purity of cell fractions by FACS analysis**

shown. Although in-vitro studies[19,20] suggest that HIV isolated from patients with advanced immunodeficiency is unable to replicate in the monocyte lineage, we have found that disease progression is associated with the spread of HIV from cells of the lymphoid system to peripheral sites such as lung, brain, and gut,[21] where many of the target cells in these tissues are of the macrophage/monocyte lineage, such as microglia in the brain.[22]

In this study, whole blood from HIV-1-positive individuals was separated into component cell populations and nucleic acid was extracted from CD4 T cells, CD8 T cells, monocytes, B cells, and dendritic cells. Limiting-dilution PCR was then used to quantify the amounts of provirus in each cell type.

Patients and methods

Blood samples

20 mL samples of whole blood were collected from 16 HIV-seropositive individuals in Edinburgh, UK. Immunological and virological information on disease progression was available for 13 of the participants, whose risk factors for infection included intravenous drug abuse and sexual contact with an HIV-positive individual. CD4 counts were available for the samples studied and for the same individuals every 1 or 2 months during the previous 6 months, so mean values could be calculated. These ranged from less than one to more than 980 CD4 lymphocytes per μL. Eight of the patients had CD4 counts associated with late-stage symptomatic disease (CD4 count <200/μL), whereas the other five had no symptoms and had CD4 counts of more than 200/μL. Absolute counts were also available for CD8 T cells, B cells, and monocytes, as well as viral p24 antigen levels.

Cell separation

CD3, CD4, CD8, CD14, and CD19 monoclonal antibodies were supplied by the Scottish Antibody Production Unit, Lanark, UK. The CD21 monoclonal was obtained from the Binding Site, Birmingham, UK, HLA-DR from American Tissue Culture Collection, Rockville, Maryland, USA, and CD11b was donated by Peter Beverley, University College, London, UK.

Blood samples were diluted with an equal volume of phosphate-buffered saline and mononuclear cells were isolated by density-gradient centrifugation (Lymphoprep, Oslo, Norway). Dendritic cells were isolated from the mononuclear cells as described previously,[6] with modifications so that other cell types could also be selected. Briefly, T cells were depleted by rosetting with neuraminidase-treated sheep erythrocytes. Magnetic beads coated with antibody to mouse IgG (Immunotech, Marseilles, France) were incubated with various mouse monoclonal antibodies, and then washed twice with phosphate-buffered saline to remove unbound antibody. T cells, which bind to the treated sheep erythrocytes (ER positive), and non-T-cell (ER negative) fractions were serially incubated on ice with these antibody-coated beads for 0·5 h and cells bound to the beads were removed by means of a magnet. These selected cells were then washed twice with the buffer.

The ER-positive fraction was incubated first with anti-CD4 to select T-helper lymphocytes and then with anti-CD8 to select cytotoxic T cells.

Cells of the ER-negative fraction were incubated with anti-CD3-coated beads to remove any contaminating T cells, and then monocytes were isolated by use of anti-CD11b and anti-CD14. B cells were depleted with anti-CD19 and anti-CD21, from the ER-negative monocyte-depleted fraction. Dendritic cells were isolated from the fraction depleted of T cells, monocytes, and B cells by use of beads coated with CD4 and HLA-DR.[23] Viable cells were counted by means of a haemocytometer, with trypan-blue stain exclusion of dead cells.

For three samples, a different method for cell separation was used. Peripheral blood mononuclear cells were incubated with anti-CD4-coated beads as before, and CD8 cells were obtained from the residual cells by use of monoclonal antibody cell sorter (MACS) anti-CD8-conjugated beads (Miltenyi Biotec Ltd, Camberley, UK).

Purity of isolated subsets

The process of isolating subsets of mononuclear cells by antibody coated magnetic beads prevented analysis of the selected cells by fluorescence-activated cell sorting (FACS) techniques. However, the purity of cell fractions could be inferred by measurement of the frequencies of different cell types in the residual cells after removal of a particular fraction. Cell populations were stained with mouse monoclonal antibodies conjugated to fluorescein isothiocyanate or phycoerythrin for FACS (Becton-Dickinson, Cowley, UK).

Quantification of HIV proviral sequences

Nucleic acid was isolated from cells and plasma by incubation in proteinase K (1 mg/mL) for 2 h at 37°C in the presence of 0·5% sodium dodecyl sulphate and poly A precipitant (40 μg/mL), followed by extraction with chloroform/phenol, and precipitation with ethanol. Proviral sequences were quantified by a previously described limiting dilution and nested PCR approach.[24] Viral genome was quantified by means of primers spanning the V3 loop and *gag* region. The nucleotide sequences of the primers and the position of the 5' base in the HXB2 genome[25] are given in table 1.

Patient number	Risk group	AIDS-defining illness	CD4 count μ(L)		CD8 count μ(L)		Monocytes (per μL)	p24 antigen (pg/mL)
			Sample used	Mean 6 mo*	Sample used	Mean 6 mo*		
26	Het/IVD	. .	<1	0·5	70	105	160	64
22	Homo	PCP	<1	0	235	348	120	87
1	IVD	PCP	2	4	571	875	270	<8
24	Het	OC	7	14	372	552	280	53
2	Het/IVD	. .	24	63	456	505	150	<8
3	Het	PCP	31	80	336	524	284	<8
27	IVD	. .	35	54	1951	2291	420	<8
4	Het/IVD	. .	162	228	646	677	190	<8
5	Het/IVD	. .	234	236	361	433	160	<8
30	IVD	. .	238	279	1244	1430	740	<8
23	Het	. .	385	305	764	764	530	<8
6	IVD	. .	959	938	2204	1422	550	112
7	IVD	. .	983	860	2102	1739	320	33

Het=heterosexual contact; IVD=intravenous drug user; Homo=male homosexual; PCP=*Pneumocystis carinii* pneumonia; OC=oesophageal candidosis.
*Mean CD4 and CD8 counts are an average of three to six results taken over 6-month period before sample was analysed.

Table 3: **Risk group, CD4 and other cell counts, and blood virus load**

Patient number	CD4 count (per μL)	Frequency of infection per 10^6 cells					
		CD4 cells	CD8 cells	Monocytes	B cells	Dendritic cells	Total PBMCs
26	<1	>483	58	4	<10*	200	8
22	<1	>4882	110	40	<10	60	167
1	2	4873	4	<4	<7·2	180	ND
24	7	44	34	<10	<20	23	4
2	24	208	24	20	<2	60	ND
3	31	39	2	<1	<2	20	ND
27	35	85	6	4	<1	<10	10
4	162	400	400	<1	<1	700	ND
5	234	40	1	<0·25	<2·5	6	ND
30	238	2	<20	<1	<10	1099	41
23	385	117	<10	<10	<10	<10	49
6	959	2	<0·05	<0·8	<1	<1	ND
7	983	11	0·5	<0·05	<0·08	40	ND

ND=not done.
*For negative samples, cut-off for assay depended on number of cells extracted.

Table 4: **Absolute cell counts and frequency of infection of 10^6 cells from different subsets of peripheral blood mononuclear cells (PBMCs) with HIV**

V3 primers provided equivalent quantification to primers in the *gag* region.[21]

Preliminary quantifications used serial ten-fold dilutions of DNA and subsequently (with V3 primers) two-fold dilutions in quadruplicate around the endpoint with between ten and 20 replicates. In addition to the selected subsets (CD4 T cells, CD8 T cells, B cells, monocytes and dendritic cells), PCR was also used to quantify the provirus load in peripheral blood mononuclear cells and the residual ER-positive populations after depletion of CD4 and CD8 cells in seven patients. Residual cells from this fraction could be unselected cytotoxic T cells or T-helper cells in which CD4 receptors were down-regulated by infection with HIV. For patients 2 and 5, up to 20 replicates of the endpoint dilution were amplified in V3 to obtain single molecules for sequencing. Quantification results were expressed as proviral copies per million cells. All separations, extractions, and amplifications were carried out with parallel samples of mononuclear cells isolated from buffy coat leucocytes derived from HIV-negative blood to serve as negative controls.

Analysis of quantification results

The relative frequencies of CD4 T lymphocytes, CD8 lymphocytes, monocytes, and B cells in the original mononuclear cell populations were estimated by the FACS method. To estimate the numbers of cells used for PCR, cell concentrations were compared before and after depletion with specific monoclonal antibodies for each subset. The only exceptions were samples from individuals with extremely low CD4 cell counts (<100/μL), where the low level of cells lost during magnetic separation greatly exceeded the numbers of selected cells, and led to a significant overestimate of the numbers of cells separated. For these samples we therefore used the absolute CD4 cell counts derived from the FACS analysis of the original blood samples to estimate cell numbers, allowing for the loss of cells during the E-rosetting step before CD4-depletion.

To ensure that all proviral sequences were accounted for by this method of analysis, we compared the total number of provirus-bearing cells from unfractionated peripheral blood mononuclear cells with the sum of each of the component subsets analysed. For example, the frequency of provirus in mononuclear cells from patient 26 was eight copies per milion cells. This was similar to the sum of the frequencies in the separate subsets. In this particular case, the contribution to the proviral sequences in the CD8 cells was 58 copies per million CD8 cells, multiplied by their frequency in the total population (70 000/mL in a total mononuclear cell concentration of 600 000/mL; 11·6%). Therefore, CD8 cells contributed 6·8 (58×0·115) cells/mL to the mononuclear cell virus load. The contributions of CD4 lymphocytes (2·1/10^6 cells), monocytes (1·1/10^6 mononuclear cells), and dendritic cells (1·3/10^6 mononuclear cells) were calculated similarly. The total of these individual contributions was 11·3 cells/mL, close to the virus load measured by PCR on unfractionated mononuclear cells (eight copies/10^6 cells). This analysis was carried out on six study individuals and yielded a total ratio of mononuclear cell proviral load to total of the subsets load of between 0·4 and 1·4. These results are consistent with the accuracy of the methods used for cell counting and quantification of HIV sequences, and independently validate the methods used for quantification of cells and provirus.

To test for correlations between proviral load in each cell type and markers of disease progression, non-parametric tests (Spearman's rank correlation test) were performed.

Results

ER-positive cells contained a mean frequency (three measurements) of 82·8% CD3 cells (table 2). Contaminating cells included B lymphocytes (0·9% CD19 cells) and natural killer cells (15·5% CD16 cells). Positive selection for CD4 cells was shown by a reduction of CD4-positive cells from a mean of 47·0% in the ER-positive population to 1·7% in the depleted cells. CD4-depleted T cells consisted of 54·5% CD8 cells, which were then positively selected on CD8-coated beads. Residual cells after CD8 depletion contained only 4·2% CD8 cells.

ER-negative cells (non-T cells) (table 2) contained less than 1% CD3-positive T cells. Monocytes were effectively removed by means of beads coated with CD11b and CD14 (59·5% to 3·7%). The remaining cells were depleted by use of CD19-coated and CD21-coated beads to remove residual B cells. Less than 1% of the resulting population expressed CD3, 43·5% CD4, and 53·2% HLA-DR, a phenotype consistent with dendritic cells.[6,23] Magnetic beads coated with anti-CD4 and anti-HLA-DR coated magnetic beads were then used to isolate the dendritic cell fraction.

The virus load in different cell types isolated from mononuclear cells of HIV-1 seropositive patients was quantified by means of limiting dilution PCR and compared with risk group, p24 antigen levels, and mean CD4 and CD8 cell counts (tables 3 and 4). We used mean CD4 cell counts taken over the previous 6 months as a marker for immunosuppression and progression to AIDS. HIV DNA was detected in the CD4 T cell subset of all 13 individuals studied (between two and more than 4882 provirus copies per 10^6 cells). Infection of non-CD4 lymphocytes was more frequent in AIDS patients (CD4 lymphocyte count <200/μL) than in non-AIDS patients. Infection was detected in monocytes of four individuals with CD4 counts of 0, 24, and 35 per μL. A similar association was found with the infection of CD8 lymphocytes, which was present in all individuals with CD4 counts less than 200/μL, whereas infection of this subset was at a low level (one virus copy per 10^6 cells, n=1) or absent (n=4) among those with CD4 counts less than

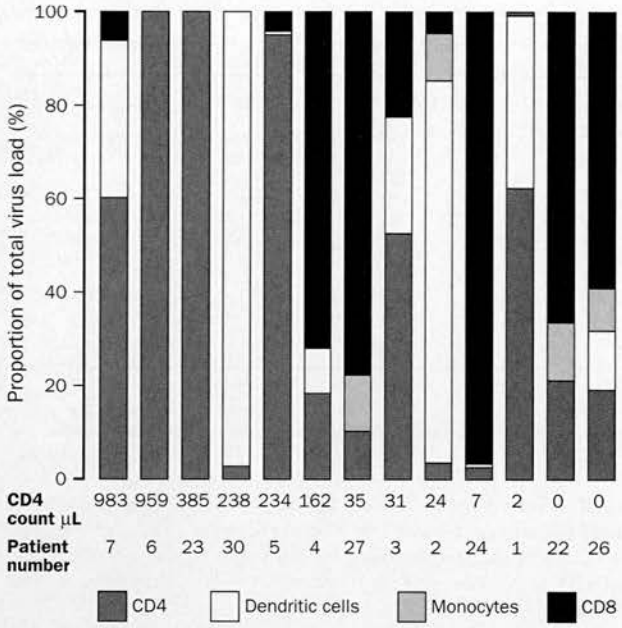


Figure: **Relative contribution of different cell subsets to proviral load in peripheral blood mononuclear cells**

Samples ranked by mean CD4 lymphocyte count, with highest value to left (table 3).

200/μL). Viral RNA was detected in plasma from ten of 13 individuals. However, DNA was never detected in plasma.

Estimations of the frequencies of infected cells, combined with their frequency in peripheral-blood mononuclear cell populations, were used to calculate the proportion of virus in each subset isolated (figure). The proportion of CD4 lymphocytes infected with HIV increased with disease progression ($p=0·004$; Spearman's rank correlation), although their contribution to total virus load in the mononuclear cell population showed a downward trend. By contrast, both the frequency of infection of CD8 lymphocytes and the proportion of HIV in the mononuclear cell population contributed by CD8 cells increased with disease progression ($p=0·04$ and $p=0·03$ respectively). There was a significant reduction in the number of circulating CD8 lymphocytes in individuals who had low CD4 counts (table 3; $p=0·005$). No correlation was found between infection of dendritic cells and monocytes with progression of disease.

Because the finding of HIV infection of CD8 cells was surprising, we separated mononuclear cell subsets from three HIV-infected individuals by a different method to isolate CD8 cells, which allowed direct analysis of their purity (table 5). First CD4 cells were removed from mononuclear cells by incubation with anti-CD4-coated magnetic beads. CD8 lymphocytes were then selected from the depleted cells using anti-CD8-coated MACS beads in a column, and these cells were used for FACS analysis and for HIV PCR. MACS beads efficiently selected CD8 cells. Frequencies of contaminating CD4 cells ranged from 0·5 to 1·3% in the three patients.

Sample number	%CD3	% Lymphocytes			Proviral copies per 10^6 cells
		CD4	B cell	%NK cells	
S04	97	1·3	0·2	2·4	1400
S05	94	1·2	0·4	0·2	84
S06	95	0·5	0·3	1·9	8·7

NK=natural killer.

Table 5: **Detection of HIV proviral sequences in cells separated by CD8-MACS beads**

Frequencies of B cells and natural killer cells were similarly low, with maximum levels of 0·4% and 2·4% respectively.

HIV proviral sequences were detected by PCR in the CD8 cells separated by MACS beads from all three patients (8·7–1400 copies per 10^6 cells; these values were similar to those obtained by the original separation method with Immunotech beads). The frequencies of proviral sequences in the cells separated by incubation with CD4 beads (containing CD4 lymphocytes, monocytes, and dendritic cells) were 128, one, and ten copies of DNA per million cells for S04, S05, and S06, respectively. Each of these frequencies was lower than those for the corresponding CD8 cell samples, indicating that no level of CD4 subset contamination of the selected CD8 cells could account for the frequency of proviral sequences shown in table 5.

The use of monoclonal antibodies from different sources in the two methods made it unlikely that cross-reactivity between the CD8 monoclonal antibody and cell-surface proteins other than CD8 was responsible for the selection of HIV-infected cells. Similarly, the very low frequencies of contaminating CD16 natural killer cells and CD4 lymphocytes made it unlikely that they were the source of HIV proviral sequences in the isolated CD8 cells.

Discussion

We found HIV infection of a range of cell types within the peripheral blood mononuclear cell populations. There was remarkable variation in the frequencies of infected cells within each subset, although there was some evidence for a consistent change in the predominant CD4 T-helper target cells upon disease progression.

A surprising finding was the frequent infection of CD8 lymphocytes from AIDS patients (as high as 400 provirus copies per 10^6 cells in one case). For methodological reasons we consider it unlikely that contamination from HIV-infected CD4 cells could have accounted for the provirus detected in CD8 cells. Levels of contaminating CD4 lymphocytes were consistently low (table 5).

We subsequently carried out sequence comparisons of the V3 region of CD8 and CD4 lymphocytes and of dendritic cells isolated from two of the patients (2, 5). Genetically distinct populations, predominantly of a predicted NSI phenotype, were found in the CD4 and CD8 cell types in both individuals, ruling out cross-contamination as an explanation for the HIV sequences detected in the CD8 lymphocytes.

Our findings tend to refute previous reports that CD8 cells were uninfected in vivo.[10,15] This discrepancy may be explained by the fact that a relatively insensitive PCR, incapable of detecting low levels of infection, was used in both of these earlier studies. However, Semensato and colleagues[26] have reported that CD8 lymphocytes from the lungs of patients with AIDS show similar frequencies of infection to those we found in the mononuclear cells.

Only four of the 13 patients tested harboured HIV provirus in peripheral-blood monocytes, consistent with previous reports of low frequencies of infection in this cell type in vivo.[10,14]

Previous reports of the infection of dendritic cells are contradictory. For example, Hsia and colleagues did not detect HIV-1 provirus in 10^4 or 10^5 dendritic cells isolated from the peripheral blood monocytes of seropositive patients,[14] whereas Patterson and colleagues detected HIV provirus by PCR in lymphocytes and purified dendritic cells of all patients investigated and found that, in each

case, the viral load was similar for both cell fractions.[18] We detected HIV-1 provirus in the peripheral blood dendritic cells of ten of the 13 patients. Independent evidence for in vivo infection of dendritic cells was obtained by sequence comparisons of the V3 region. Distinct populations of HIV-1 variants were found in dendritic cells compared with those found in CD8 and CD4 cells (data not shown).

Natural killer cells, which we found to be present in ER-positive cells (15·5%; table 2) express low levels of the CD8 receptor and can be infected with HIV in vitro.[27] However, low frequencies of natural killer cells were consistently found in the CD8-positive population selected by MACS beads (table 5). Most cells expressed CD3 and a maximum of 2·4% expressed CD16. Natural killer cells are therefore unlikely to be the source of the HIV proviral sequences detected in the CD8 population.

Although CD8 lymphocytes do not express CD4, in vitro infection of CD8 lymphocytes with HIV-1 has been achieved by co-culture with HIV-1-infected CD4 cells.[28] The interaction between CD4 and CD8 cells occurring in vivo as part of the immune response may transmit infection to CD8 lymphocytes. An alternative explanation is suggested by studies in SCID mice (ie, animals with severe combined immunodeficiency), which indicate that infection of human progenitor T cells may occur in the thymus of infected animals during normal selection for both antigen specificity and co-receptor (CD4 or CD8) expression.[29] Thus the peripheral CD8 cells we found to be infected with HIV could have taken up virus within the thymus while expressing CD4 during normal selection at an immature stage. This hypothesis would explain why we only see infected CD8 cells later in disease. An alternative explanation for widespread infection of CD8 cells is that HIV can infect cells through a non-CD4-dependent mechanism of virus attachment and entry. CD8 lymphocytes, in common with CD4 lymphocytes and macrophages, express the CC-CKR-5 receptor (M Dittmar, personal communication), which has been identified as a second receptor for non-syncytium-inducing variants of HIV-1.[30,31] Infection of CD8 lymphocytes may therefore occur through a different as yet unidentified atachment protein, but there may be a common pathway for viral phenotypes in cell fusion and entry. Our current understanding of the tropism of HIV-1 is largely derived from in-vitro investigation of the properties of virus isolates. The more restricted tropism of these isolates for CD4 cells could result from selection. For example, the process of virus isolation and co-culture with primary lymphocytes could preferentially stimulate CD4-tropic variants at the expense of variants with tropisms for cell types not activated by phytohaemagglutinin. Most laboratory strains of HIV lose their ability to replicate in macrophages. Current methods of virus culture may also select against variants that can replicate in certain cell types such as CD8 cells in which a different cellular receptor is used.

One of the factors contributing to the high frequency of CD8-positive lymphocytes occurring only in late-stage disease is loss of the CD4 T-cell population from circulation. On the other hand, a substantial increase in CD8 T-cell infection on disease progression may possibly be related to the phenomenon by which HIV spreads to non-lymphoid tissues during the later stage of disease,[21] with loss of immune control.

These results are also consistent with the possibility that the decline in CD8 T cells may be a direct consequence of the cytopathic effect of HIV on this subset and that HIV has a broader cellular tropism than previously described. These findings have important implications for understanding of the pathogenesis of HIV and the design of intervention studies.

Edinburgh Heterosexual Transmission Group

Participating institutions and investigators: J Whitelaw, HIV Immunology, Royal Infirmary of Edinburgh; R Wyld, J R Robertson, Muirhouse General Practice; and R P Brettle, Regional Infectious Diseases Unit, City Hospital.

Acknowledgments

We thank staff in the Department of Neuropathology and Medical Microbiology and the Centre for HIV Research, Edinburgh University; E Hughes and J Cresswell for technical help; D Smith for discussion; the Heterosexual Transmission Study (Medical Research Council grant FPG 9102644) for supply of samples; John Hanley (Department of Haematology), and Helen Mason (HIV Immunology, Royal Infirmary of Edinburgh), for clinical and laboratory data; and Robin Weiss and Myra McClure for valuable discussion of the research findings.

This work was supported by a Medical Research Council grant PG 9209918.

References

1 Levy JA. Pathogenesis of human immunodeficiency virus infection. *Microbiol Rev* 1993; **57:** 183–289.

2 Macatonia SE, Lau R, Patterson S, Pinching AJ, Knight SC. Dendritic cell infection depletion and dysfunction in HIV-infected individuals. *Immunology* 1990; **71:** 38–45.

3 Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 1984; **312:** 763–67.

4 Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* **1986;** 47: 333–48.

5 Klatzmann D, Champagne E, Chamaret S, et al. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* 1984; **312:** 767–68.

6 O'Doherty U, Steinman RM, Peng M, et al. Dendritic cells freshly isolated from human blood express CD4 and mature into typical immunostimulatory dendritic cells after culture in monocyte-conditioned medium. *J Exp Med* 1993; **178:** 1067–76.

7 Weissman D, Li YX, Ananworanich J, et al. Three populations of cells with dendritic morphology exist in peripheral blood, only one of which is infectable with human immunodeficiency virus type 1. *Proc Natl Acad Sci USA* 1995; **92:** 826–30.

8 Collman R, Godfrey B, Cutilli J, et al. Macrophage-tropic strains of human immunodeficiency virus type 1 utilize the CD4 receptor. *J Virol* 1990; **64:** 4468-76.

9 Valentin A, von Gegerfelt A, Matsuda S, Nilsson K, Asjo B. In vitro maturation of mononuclear phagocytes and susceptibility to HIV-1 infection. *J Acquir Immune Defic Syndr* 1991; **4:** 751–59.

10 Schnittman SM, Psallidopoulos MC, Lane HC, et al. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. *Science* 1989; **245:** 305–08.

11 Innocenti P, Ottman M, Leclercq P, Seigneurin JM. HIV-1 in blood monocytes: frequency of detection of proviral DNA using PCR and comparison with total CD4 count. *AIDS Res Hum Retroviruses* 1992; **8:** 261–68.

12 Bagasra O, Pomerantz RJ. Human immunodeficiency virus type I provirus is demonstrated in peripheral blood monocytes in vivo—a study utilizing an in situ polymerase chain reaction. *AIDS Res Hum Retroviruses* 1993; **9:** 69–76.

13 Schrier RD, McCutchan JA, Wiley CA. Mechanisms of immune activation of human immunodeficiency virus in monocytes/macrophages. *J Virol* 1993; **67:** 5713–20.

14 Hsia K, Tsai V, Zvaifler NJ, Spector SA. Low prevalence of HIV-1 proviral DNA in peripheral blood monocytes and dendritic cells from HIV-1-infected individuals. *AIDS* 1995; **9:** 398–99.

15 Psallidopoulos MC, Schnittman SM, Thompson LM, et al. Integrated proviral human immunodeficiency virus type 1 is present in CD4+ peripheral lymphocytes in healthy seropositive individuals. *J Virol* 1989; **63:** 4626–31.

16 Chehimi J, Prakash K, Shanmugam V, et al. CD4-independent infection of human peripheral blood dendritic cells with isolates of human immunodeficiency virus type-1. *J Gen Virol* 1993; **74:** 1277–85.

17 Cameron PU, Pope M, Gezelter S, Steinman RM. Infection and apoptotic cell death of CD4+ T cells during an immune response to HIV-1-pulsed dendritic cells. *AIDS Res Hum Retroviruses* 1994; **10:** 61–71.

18 Patterson S, Roberts MS, English NR, et al. Detection of HIV DNA in peripheral blood dendritic cells of HIV-infected individuals. *Res Virol* 1994; **145:** 171–76.

19 Tersmette M, de Goede REY, A1 BJMM, et al. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J Virol* 1988; **62:** 2026–32.
20 Cheng-Mayer C, Levy JA. Distinct biologic and serologic properties of HIV isolates from the brain. *Ann Neurol* 1988; **23:** s58–s61.
21 Donaldson YK, Bell JE, Holmes EC, Hughes ES, Brown HK, Simmonds P. In vivo distribution and cytopathology of variants of HIV-1 showing restricted sequence variability in the V3 loop. *J Virol* 1994; **68:** 5991–6005.
22 Watkins BA, Dorn HH, Kelly WB, et al. Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science* 1990; **249:** 549–53.
23 Freudenthal PS, Steinman RM. The distinct surface of dendritic cells, as observed after an improved isolation method. *Proc Natl Acad Sci USA* 1990; **87:** 7698–7702.
24 Simmonds P, Balfe P, Peutherer JF, Ludlam CA, Bishop JO, Leigh Brown AJ. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells at low copy numbers. *J Virol* 1990; **64:** 864–72.
25 Myers G, Korber B, Berzofsky J, Smith TF, Pavlakis GN, eds. Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos, New Mexico: Los Alamos National Laboratory 1993.
26 Semensato G, Agostini C, Ometto L, et al. CD8(+) t lymphocytes in the lung of acquired immunodeficiency syndrome patients harbor human immunodeficiency virus type 1. *Blood* 1995; **85:** 2308–14.
27 Chehimi J, Bandyopadhyay S, Prakash K et al. In vitro infection of natural killer cells with different human immunodeficiency virus type 1 isolates. *J Virol* 1991; **65:** 1812–22.
28 De Maria A, Pantaleo G, Schnittman SM, et al. Infection of CD8+ T lymphocytes with HIV. Rquirements for interaction with infected CD4+ cells and induction of infectious virus from chronically infected CD8+ cells. *J Immunol* 1991; **146:** 220–26.
29 Su L, Kaneshima L, Bonyhadi M, et al. HIV-induced thymocyte depletion is associated with indirect cytopathicity and infection of progenitor cells in vivo. *Immunity* 1995; **2:** 25.
30 Deng H, Liu R, Elleneier, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996; **381:** 661–66.
31 Dragic T, Litwin V, Allaway GP, et al. HIV-1 entry in CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996; **381:** 667–73.

White-coat hypertension as a cause of cardiovascular dysfunction

Stephen K Glen, Henry L Elliott, Joan L Curzio, Kennedy R Lees, John L Reid

Summary

Background The increasing use of 24 h ambulatory blood pressure monitoring has allowed diagnosis of white-coat hypertension, in which blood pressures are higher on clinic measurements than on ambulatory monitoring. Treatment is not generally thought to be necessary for this disorder. However, there is evidence that patients with white-coat hypertension develop renal impairment and left ventricular hypertrophy. We undertook this study to assess whether white-coat hypertension, in the absence of cardiovascular structural abnormalities, is associated with cardiovascular functional abnormalities.

Methods Cardiovascular function was assessed by ultrasonography in three groups of patients classified as normotensive, persistently hypertensive, or white-coat hypertensive (23, 20, and 22 patients, respectively) on the basis of ambulatory blood pressure monitoring, carried out for 28 h with recordings taken every 15 min during the day and every 20 min during the night, and clinic measurements, made with a semi-automatic oscillometric device.

Results Similar abnormalities of diastolic left ventricular function were identified in the patients with persistent hypertension and those with white-coat hypertension; both groups differed in these indices from the normotensive group (E/A ratios 0·94 [SD 0·23], 1·06 [0·21], and 1·24 [0·31] respectively; ANOVA $p<0.005$). In addition, the white-coat and persistently hypertensive groups, when compared with the normotensive group, showed similar abnormalities of elasticity, compliance, and stiffness (stiffness index 4·32 [1·90], 4·53 [1·38], and 3·27 [0·95] respectively; ANOVA $p<0.05$) of the large arteries.

Interpretation Functional cardiovascular abnormalities were identified in white-coat hypertensive patients who had no identifiable structural abnormalities. Such functional abnormalities can be reversed by antihypertensive treatment. We propose that patients with white-coat hypertension might benefit from antihypertensive treatment as well as those with persistent hypertension. This hypothesis should be addressed in prospective clinical trials.

Lancet 1996; **348:** 654–57
See Commentary page 630

Department of Medicine and Therapeutics, Western Infirmary, Glasgow, UK (S K Glen MRCP, H L Elliott MD, J L Curzio PLD, K R Lees MD, J L Reid DM)

Correspondence to: Dr Stephen Glen, Department of Cardiology, Western Infirmary, Glasgow, G11 6NT, UK

Introduction

Most clinical trials of antihypertensive treatment have relied on conventional clinic measurement of blood pressure to identify hypertensive patients and to demonstrate the benefits of blood pressure reduction.[1] However, 20–40% of individuals with borderline clinic hypertension can be reclassified as normotensive during daytime ambulatory monitoring,[2–5] and these trials inevitably included some such individuals with white-coat hypertension. However, controversy remains about the precise definition of white-coat hypertension. For example, one definition is a raised diastolic pressure (90–104 mm Hg) at clinic but normal daytime ambulatory blood pressure (below the 90th percentile of a normotensive control group)[2] and another is a mean clinic blood pressure at least 6 mm Hg higher than the ambulatory mean.[3] Nevertheless, the inclusion of white-coat hypertensive patients in the studies cited did not prevent the demonstration of significant reductions in cardiovascular disease. Irrespective of the definition, and despite the recognition that the presence of medical personnel influences blood pressure,[6] the general assumption is that the inclusion of white-coat hypertensive patients may have diluted the overall magnitude of the benefits of a drug treatment found in previous studies. Another possibility is that the white-coat hypertensive patients also benefited from active treatment—this idea is the substance of our hypothesis. The main aim of our pilot study was the non-invasive measurement of a range of indices of cardiovascular function in patients referred for evaluation of their hypertension, to find out whether patients with white-coat hypertension differ from patients with hypertension of